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Formulated Delivery of Enzyme/Prodrug and Cytokine Gene Therapy to Promote Immune Reduction of Treated and Remote Tumors in Mouse Models of Prostate Cancer

Annual Report, January, 2005. DAMD17-02-1-0107

INTRODUCTION:

Prostate cancer (PC) is now the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery and radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatment strategies are needed. The **subject** of this work is a study of gene therapy, used alone and in combination with hormones called cytokines that stimulate the immune system. These genes will be delivered using lentiviral or adenoviral vectors. *The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CDUPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5FC), is then given, cancer cells producing CDUPRT convert 5FC to a toxin that kills the original cell and others nearby. This strategy is suitable for slow growing tumors like PC. Killing the tumor cells attracts immune cells. The **scope** of the work involves preparation of the gene vectors, optimizing the conditions required for delivering the genes of interest using viral vectors, and identification of the immune cells that infiltrate the tumor when gene therapy is used. Having identified these cells, we will use cytokine genes delivered into the tumor to attract more of them to this site. We will then compare the effects of delivering the cytokine gene therapy alone, the suicide gene therapy alone, or a combination of both into mice that carry a murine prostate cancer cell line, RM1 cells, grown in the prostate. We predict that the combination therapy should interfere with the growth of the cancer cells in the prostate and should also cause a reduction in the number and extent of tumor cells that grow in the lung after introduction into the mice via intravenous injection. This work should pave the way for clinical trials of combination therapy involving suicide gene therapy and cytokine gene therapy given together into the prostate of men with PC.

BODY:

New cell lines have had to be prepared for the studies described below. In addition, we have had to prepare and characterize both plasmids, adenoviral and lentiviral vectors containing the genes of interest for delivery into PC. Not only have we manufactured the plasmids and recombinant viral vectors for delivery of the genes of interest, but we have also prepared stable transfectants from a murine PC cell line, the RM1 line, kindly provided by Dr T Thompson, Baylor College, Texas. The use of transfected cell lines will allow us to generate a maximum effect *in vivo*, and we will then be able to compare the possibilities that can be generated using a viral vector as the delivery vessel. In the first instance, the work was based on our previous studies that showed that the gene, purine nucleoside phosphorylase (PNP), could be used for gene-directed enzyme prodrug therapy (GDEPT) directed against PC (Martiniello *et al.*, 1998; Martiniello-Wilks *et al.*, 2002; Voeks *et al.*, 2002a). However, due to problems with intellectual property, we were no longer able to use this gene, and moreover, workers from CSIRO decided not to be involved in the ongoing work. Instead, we have recruited Dr Aparajita Khatri, PhD (starting August, 2003) Dr Bing Zhang, PhD (started November, 2003, but left June 2004) and Ms Eboney Doherty, Bsc. Hons, who started in July, 2003. Unfortunately, Dr Zhang left us after 6 months for unforeseen family reasons. We subsequently recruited Ms Jane Chapman for technical help. She started work in April, 2004). Instead of using PNP, we have chosen the fusion gene, CDUPRT for the following reasons: CD

converts 5 fluorocytosine to 5 fluorouracil, whose metabolites, 5-fluoro-2'-deoxyuridine 5'-monophosphate (5FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) damage DNA and RNA respectively. The rate-limiting step in the generation of 5FdUMP and 5FUTP is the formation of an intermediary metabolite, 5-fluorouridine mono-phosphate (5FUMP). 5-FUMP is only produced after a series of catalysed enzymatic reactions. This can be circumvented by the ability of UPRT to convert 5FU directly to 5FUMP thereby leading to more efficient production of anti-tumor metabolites, 5FdUMP and 5FUTP (Tiraby *et al.*, 1998). UPRT sensitizes cancer cells to low doses of 5FU (Kanai *et al.*, 1998), and when used in conjunction with CD and 5FC in GDEPT, was more effective than CD-GDEPT alone against colon cancer (Koyama *et al.*, 2000; Chung-Faye *et al.*, 2001) and glioma (Adachi *et al.*, 2000) *in vitro* and *in vivo*. There are no reports of this combination (CDUPRT) being used against PC, making our application novel. Thus *drugs generated by CDUPRT can kill both dividing and non-dividing cells*. This is important in PC, where the percentage of dividing cells is low. Moreover, *metabolites of 5 fluorocytosine can produce a local bystander effect* (Adachi *et al.*, 2000; Pierrefite-Carle *et al.*, 1999) and finally, *CD-GDEPT has been shown to generate a distant bystander effect* against colon carcinoma of the liver that was largely mediated by natural killer cells (Pierrefite-Carle *et al.*, 1999).

During the last 12 months, we have implanted RM1 PC cells stably transfected with CDUPRT in the prostate of C57BL/6 males, and treated the mice with 5FC. We now wish to compare the killing obtained using this system with that generated by the PNP gene that we have previously used. For this reason, we are in the process of preparing lentiviral vectors that express PNP in addition to Lent.CDUPRT, for comparative purposes.

As we have generated PC cells transfected with the cytokine genes of interest, we are now in the position to compare the results of directly injecting these transfected cells into the mice, or using lipid-plasmid combinations or lentiviral vectors, which may be less efficient, but which would be more likely to have direct application in clinical studies.

DOD Alternate: The work was late in starting because of the intellectual property considerations, and the changes that were necessary to the program. The new program Statement of Work, accepted by the DOD, was shown in Appendix 1 of our Annual Report for 2003 (January, 2004). New staff were recruited, as described above, and because of this, the work could not be commenced until July, 2003. The current report represents a total of 18 months of work.

Task 1:

GDEPT alone. Assess the ability of lentivirus expressing GDEPT (based on the fusion gene, cytosine deaminase/uracil phosphoribosyltransferase (CD/UPRT) to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 1-12)

a) Prepare recombinant lentivirus

Methods and Results:

Plasmid preparation and characterization: CDUPRT was obtained in the pORF-codA::upp plasmid from Invivogen (San Diego, CA, USA). The CodA::upp(CDUPRT) gene was excised from this plasmid using the *NcoI* and *NheI* restriction enzyme sites and ligated into complementary sites in the pVITRO2-GFP/LacZ expression plasmid (See Jan 2004 report, Appendix II, Figure 1). pVITRO2-GFP/LacZ, which also contains the genes for the jelly fish green fluorescent protein, GFP, and for the bacterial enzyme, β galactosidase, LacZ, that can be used as reporter genes to monitor the progress of the preparation of plasmids, was used as a control vector and cell line throughout these experiments and also provided the backbone plasmid for insertion of all genes of

interest. The resulting pVITRO2-GFP/CDUPRT plasmid was characterized for the presence of the genes of interest by restriction enzyme digestion using *NcoI* and *NheI* (Jan 2004 report, Appendix II, figure 1).

Virus preparation and characterization:

The recombinant lentiviruses to be constructed and used for this study are Lenti.CMV.CDUPRT (GDEPT) and Lenti.CMV.GFP. The GFP expressing virus will serve as a control virus for all the downstream experiments.

The virus construction is being carried out according to the instructions in the ViraPower™ lentiviral expression system kit (Invitrogen, California, USA). The general strategy for the virus construction is as follows:

The pLenti-based expression vector and the packaging mix will be co-transfected into the 293FT cell line (INVITROGEN) to produce a lentiviral stock. The recombinant lentiviral stock will then be purified by ultracentrifugation and the purified stocks titred for use in the proposed experiments.

Construction of pLenti.CDUPRT expression construct:

Gateway technology developed by INVITROGEN was used for construction of the expression clone (see schematic representation of protocol). This technology is based on the capacity of the bacteriophage lambda for site-specific recombination system facilitating the integration of lambda into the *E. Coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1992). For our purposes, the components of the lambda recombination system are modified to improve the specificity and efficiency of the system (Bushman *et al.*, 1985).

1. Recombination occurs between specific attachment (*att*) sites on the interacting DNA molecules. Recombination is conservative (*i.e.* there is no net gain or loss of nucleotides) and requires no DNA synthesis. Strand exchange occurs within a core region that is common to all *att* sites. Briefly, the construction of the transfer vector involves the following steps:
2. Entry clone containing the gene of interest flanked by the *att* sites is constructed. This entry clone is then recombined with the destination vector (pLenti6/V5-DEST; INVITROGEN) using the Clonase enzyme mix (Invitrogen) in an *in vitro* recombination reaction. This would yield the recombinant lenti-expression vector expressing the transgene for generation of the recombinant virus as described above.

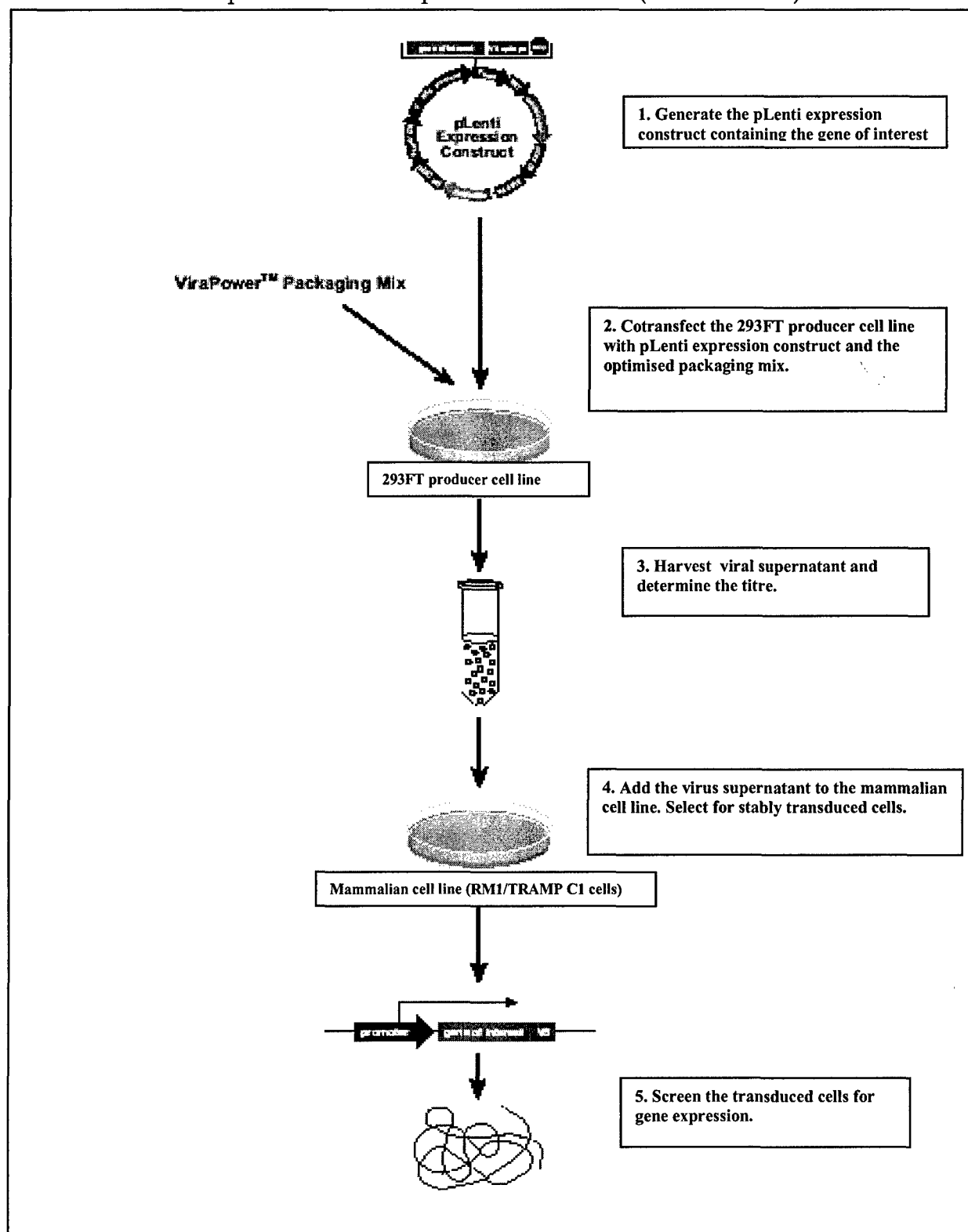
Step 1: Construction of the entry vector containing CDUPRT or GFP genes (pENTR1A-CDUPRT; pENTR1A-GFP): The entry vector (pENTR 1A; Invitrogen), especially designed for use in downstream recombination reactions was propagated in DB3,.1 strain of E.Coli.

The CD-UPRT or GFP genes were PCR amplified from pORF-codA::upp (Invivogen;San Diego, CA, USA) and pVITRO2-GFP-LacZ plasmids (Invivogen;San Diego, CA, USA), respectively (Jan 2004 report, Appendix II, figure 2A). To ensure the fidelity and integrity of the amplification, *Pfu* TURBO DNA polymerase (Stratagene; California, USA) was used for the PCR amplification.

The primers were designed (Appendix IV) such that the CDUPRT or the GFP sequence

- 1.contained an ATG codon in the context of a KOZAK translation sequence (G/A)NNATGG) for proper initiation of translation in mammalian cells (Kozak,1987; Kozak, 1990; Kozak, 1991).
- 2.was in frame with the C-terminal tag after recombination with destination vector.
- 3.did not contain a stop codon for expressing the CDUPRT protein combined with C-terminal tag (V5) in the destination vector. This would be useful for antibody-based detection of the vector in downstream applications.

A schematic representation of the protocol is as follows (INVITROGEN):



Once the genes were successfully amplified with the necessary modifications (Jan 2004 report, Appendix II, figure 2A), the fragments were gel purified (Gene clean spin kit; BIO101inc, CA, USA) and digested with restriction endonucleases (*Bam*HI and *ECOR*I) followed by another gel purification. These fragments were then ligated to gel purified *BAM*HI and *ECOR*I digested pENTR vector, using T4 DNA ligase. The ligated product was used to transform chemically competent TOP 10 *E. Coli* strain (Invitrogen), which were then selected under kanamycin selection. The positive clones were screened using the appropriate restriction enzymes for digestion (Jan 2004 report, Appendix II, figure 2). *We have successfully generated pENTR-CDUPRT and pENTR-GFP.* Integrity of the entry clones will be further confirmed by sequencing. The primers have been designed (Appendix II) and clones have been sequenced (data not shown). The sequence data was not very reliable due to error in sequencing, hence the pENTR.GFP and pENTR.CDUPRT constructs were analysed primarily using the restriction digests (January 2005 report, Appendix II, **Figure 1**) and subsequently, the viruses rescued using these constructs were function tested (see below, step 3)

Step 2: Recombination of pENTR-CDUPRT or pENTR-GFP with the destination vector containing the Lentiviral elements (pLenti6/V5-DEST):

The protocol followed for the recombinations are in accordance with the instructions in the supplier's kit manual (Invitrogen). The destination vector, pLenti6-V5DEST (Invitrogen) was propagated in DB3.1 *E. Coli* strain (Invitrogen). Briefly, 300 ng of entry clone was recombined with 300 ng of destination vector in an *in vitro* reaction using LR Clonase enzyme mix (Invitrogen). The reaction was carried out at room temperature for 18 hr. At the end of the incubation the reaction was stopped by ten minute incubation with 4µg of proteinase K/ reaction at 37°C. LR recombination mix (4 µl) was transformed into TOP10 strain of *E. Coli*. The clones were selected under Ampicillin (100 µg/ml) and Blasticidin (50µg/ml) selection. The clones positive for pLenti6/V5-CDUPRT were screened using restriction endonucleases *Eco*R1 and *Kpn*I (January 2005 report, Appendix II, **Figure 1A**). Recombinations were successfully done for the pLenti6/V5GP and the transformed clones were screened with KPN1.(January 2005, Appendix II, **Figure 1B**). The DNA from the positive clones (pLenti6/V5-CDUPRT) was finally propagated in HB101 strain of *E. Coli*.(see below) for use in rescue of the recombinant viruses according to the manufacturer's protocol (see above).

Propagation of plent.CDUPRT and pLent.GFP for subsequent use in virus rescue:

The glycerol stock (Top 10 cells) of the recombinant clones were streaked on agar plates with Blasticidin (50µg/ml) and ampicillin (200µg/ml). 12 colonies were picked and screened for the positive clones using KPN1 digest for the plentGFP and KPN1 and *Eco*R1 digest for the plent.CDUPRT clones. The DNA from one positive clone from each set (#4 for CDUPRT and #6 for GFP) was then used to transform HB100 cells and a either 500 mls of terrific broth (TB) or L broth (LB) was inoculated directly using a single colony from the plate. Glycerol stocks were made from the megaprep cultures and stored at -70°C. The DNA was prepared using the endotoxin-free Qiagen Maxi kit.

A summary of this work is provided in Table 1.

Step 3: Rescue of the Lent.CDUPRT and Lent.GFP using 293FT cells:

The purified DNA (representing the transfer vectors) prepared in step 2 was then used for the rescue of the respective viruses using a four-plasmid transfection system (according to the instructions from the suppliers). The transfer vectors were co-transfected into 293FT cells (Invitrogen) with the packaging mix comprising three plasmids, which supply the viral proteins *in trans* that are required for generation of the transgene containing Lentiviral particles (Naldindi et al., 1996).

Table 1: Summary of bulk preparation of pLentiviral purified DNA:

No.	Date	Name	Treatment	Ratio (260/280nm)	Concentration	Total Volume.
1.	1.6.04	pLenti6.V5.GFP Clone#6	LB (250ml)	1.74	400µg/mL	400µL
2.	4.6.04	pLenti6.V5.CDUPRT Clone#4	TB (250ml)	1.77	1.64 mg/mL	400µL
3.	4.6.04	pLenti6.V5.CDUPRT Clone#6	LB (250ml)	1.77	410µg/mL	400µL
4.	18.6.04	pLenti6.V5.GFP Clone#6	TB (250ml; Amp+Blastcidin)	1.82	2.00mg/mL	400 µL
5.	18.6.04	pLenti6.V5.GFP Clone#6	TB (250ml;Amp only)	1.84	3.42mg/mL	400µL

Since generation of high yields of the virus is reliant upon the efficiency of the transfection, the transfection was optimised to get high yields of the viruses. High yield of the viruses is essential to successfully carry out the *in vivo* experiments. This optimization was done using pLent.GFP, as GFP expressing cells can be easily monitored by UV microscopy and flow cytometry, thus allowing accurate analysis of data, both qualitatively and quantitatively.

Optimization of the transfections:

Two transfection methods were tested for their efficiency:

1. *Lipofectamine based transfection:* The Lipofectamine 2000 reagent supplied with the kit (Ciccarone et al., 1999) was used according to the instructions from the suppliers (Invitrogen). It is a cationic lipid based formulation suitable for the transfection of the nucleic acids into eukaryotic cells. Briefly, transfer DNA and the packaging mix were first complexed with the transfection reagent and then added to the plates containing the growth media. The 293FT cells were then added to the media containing the DNA-Lipofectamine complexes. The cells are allowed to attach and transfect overnight. The growth media was changed the next day and rescued virus was collected in the supernatant at 48 and 72 h post transfection. The cell debris from the virus containing supernatants was removed using 45micron filters and the supernatants were stored at -80°C . Alternatively, cells were replated after overnight transfections and viral supernatants were collected at 48 and 72 h post-transfection.
2. *Calcium Phosphate based transfection (CaP):* This was a modification of calcium phosphate method of transfection described by Graham and Van der Eb (1973). Briefly, 293FT cells were transfected at 80-90% confluency with DNA-calcium complexes (100 mM dish). On the day of transfection, transfer vector and packaging mix plasmids were mixed in a polystyrene tube with final volume of up to 500 µL of 250mM CaCl_2 (made in 1/10 of low Tris EDTA (1mM Tris, 0.1 mM EDTA, pH 7.6). To this was added an equal volume of 2x HBS (Hepes buffered saline, 140mM NaCl, 1.5 mM NaPO_4 , 50 mM Hepes, pH 7.05). The mix was incubated for 30 minutes then added to the culture media on cells. Next day, the media were replaced with the fresh media or the cells were replated. The virus containing supernatant was harvested at 48 and 72 h post transfection and stored at -80°C after passing through a 45 Micron filter.

The efficiency of the transfection was measured by Flow Cytometric analysis of green fluorescent cells 24 h post transfection. The efficiency of the transfection was much higher for CaP (16% green cells) in comparison with Lipofectamine 2000 based transfection (11%) (Data not shown).

Functional test of Lent.GFP:

The supernatants obtained from transfected supernatants were analyzed for the presence of recombinant viruses by analyzing the transduced 293 cells for gene expression 72 h later. The green fluorescent 293 cells transduced with Lent.GFP supernatant could be detected under the UV microscope and by flow cytometry. This was also used to establish the viral titres obtained from the transfection optimisation experiments (January 2005 report, Appendix II, **Figure 2**). The data obtained clearly showed that CaP transfection was a more efficient method for virus production (titres upto 1.2×10^6 transducing units (TU) /ml, 20 ml/100mM dish) in comparison to Lipofectamine 2000 based transfection (4.2×10^5 TU/ml, 20 ml/100mM dish). Further, the data clearly indicated that replating the cells the next day after transfection led to almost 4 fold increase in production of the recombinant viruses (January 2005 report, Appendix II, **Figure 2**). Optimization experiments are currently underway to further improve the viral titres.

Functional test of Lent.CDUPRT:

Lent.CDUPRT supernatants were used to transduce 293A cells at three different doses (100, 10 and 1 μ L/well) for 48 h and then the cells were treated with 5-fluorocytosine at 1mM concentration. 72 h post treatment, the cells were analysed for proliferation using WST1 reagent and absorbance was measured at 450nm. 293 cells transduced with Lent.CDUPRT with no 5FC and 293 cells transduced with the control virus Lent.GFP in the presence of 5FC served as the controls for this experiment. The data obtained clearly indicated that Lent.CDUPRT is functional (January 2005 report, Appendix II, **Figure 3**)

We now have the functional Lentiviral vectors and are in an excellent position to carry out the subsequent in vivo experiments.

TASK 1b. Establish conditions for implanting TRAMP-C1 cells subcutaneously in transgenic TRAMP mice.

The aim of this experiment was to determine the take rate of implanted TRAMP C1 tumor cells in TRAMP (transgenic adenocarcinoma mouse prostate) mice, in order to establish the model for the future experiments as proposed in this study. TRAMP C1 is a murine PC cell line kindly provided by N Greenberg, USA, from spontaneously arising tumors from the TRAMP mice. Seven TRAMP mice were injected sc with 5×10^6 TRAMP C1 cells. This was on the basis of the take rate of TRAMP C1 cells determined in C57BL/6 mice in our laboratory (Voeks et al., 2002b). *Tumor growth wasn't detected until 2 months after implantation.* Tumor samples were harvested and stored at -80°C in OCT or were formalin fixed and paraffin-embedded for storage at room temperature. Examination of H&E stained sections of these samples showed that the morphology of spontaneous tumors ranged from poor to well differentiated. January 2004 report, Appendix II, **figure 3** shows an example of relatively differentiated tumor as shown by the glandular architecture of these tumors. The morphology of the subcutaneous TRAMP C1 tumors, by comparison, was consistent with poorly differentiated and invasive tumors, showing loss of glandular architecture (Jan 2004 report, Appendix II, **figure 3**). This could reflect the fact that the TRAMP C1 cell line is derived from a poorly differentiated tumor.

In addition, TRAMP C1(TC1) and C2 (TC2) cell lines were grown *in vitro* in the absence of androgen (charcoal stripped FBS) to generate androgen-refractory cell lines, or were inoculated sc into female TRAMP mice. From these studies, we have isolated 7 androgen-refractory TRAMP cell lines from the TRAMP C1 and C2 cell lines, and these are currently being characterized for

androgen-receptor status, growth *in vitro* and *in vivo*, as described in the poster presented at Prince of Wales Hospital, September, 2004 for the "TOW" prize. (Jan 2005 report, Appendix III). Their growth *in vivo* is shown in Appendix II, **Figure 4**.

TC1-T3 and TC2-T3 cells were of particular interest as they grew in female mice and the growth rate was the same as the parental cells in male mice. Further, TC1-F1 cell line (derived from a primary culture from a TC1 tumour grown in female mice) grew faster in females (tumor onset at week 2) compared to the males (tumor onset at week 3) suggesting an inhibitory effect of androgen on tumor growth in males. These cell lines are currently being characterized with regards to their androgen receptor status and *in vivo* growth properties such as metastatic potential and invasiveness.

The derivation and characterization of these androgen independent cell lines from the TRAMP tumors will help expand our in vivo model to encompass all stages of prostate cancer. This will broaden the scope of the future experiments.

TASK 1c Optimize dose of virus needed to establish GDEPT in orthotopically implanted RM-1 tumors when formulated with plasmid.

Before performing this task, we optimized the CDUPRT-GDEPT + 5FC system and determined the following:

i.) Optimization of dose of 5FC in vivo, and examination for toxic effects.

This was described in our January 2004 report under section c (i). We found that C57BL/6 mice could tolerate 5FC at a dose up to 500 mg/kg/mouse, given intraperitoneally (ip) every day for 13 days without systemic toxicity as observed by serum analysis (See January 2004 report, Table 1) or by histological examination of major organs (January 2004 report, Appendix II, **figure 4**)

ii) Assessment of CDUPRT expression: To assess CDUPRT expression, we first stably transfected RM-1 cells with GFP and CDUPRT as described in our January 2004 report, section c (ii), using hygromycin B for selection at 800 µg/mL of culture medium. Control cells were stably transfected to express GFP alone or GFP/LacZ, and were known as RM1-GFP or RM1-GFP/LacZ (controls) respectively. The green fluorescence protein (GFP) was used as a marker to follow the cells *in vivo*, and to sort high expressers as described previously (see Jan 2004 report, Appendix II, **Figures 5, 6, 13**).

CDUPRT expression by transfected cells:

In vitro: The stably transfected RM1-GFP/CDUPRT cells were examined to show that the genes of interest were functional. An HPLC based assay tested the capability of the cell/ lysates to catabolize the prodrug 5FC to 5FU. Specifically, 10⁶ RM1-GFP/CDUPRT /RM1-GFP/LacZ cells were resuspended in 110 µL of PBS and lysed by repeat freeze thawing of the cells by alternating between incubation in liquid Nitrogen and a 37⁰ C waterbath. Cell debris was removed by centrifugation of the lysates at 12,000g for 10 minutes. The protein content of the supernatants was determined using the BCA protein estimation assay kit (Pierce, Rockford, IL, USA). 100µL of the supernatant was then incubated with 900µL of 0.5 mM 5FC at 37⁰ C (waterbath) for different time periods (1, 6.5 and 24 h). At each time point 100µL of the samples were harvested and incubated at 85⁰ C for 10 minutes, then stored at -20⁰ C until analysis. The samples were analysed using reverse phase chromatography. Sample (10µL) was injected into a C18 column under isocratic conditions (0.05% Trifluoroacetic acid in water) at a flow rate of 0.7 mLs/min. Absorbance was measured at 275 nm. The enzyme activity for each sample was determined by ratios of the peak areas for 5FC

and 5FU. High enzymic activity was measured over a 24 h period (Jan 2005 report, Appendix II, **Figure 5A**). These data confirmed our previous findings (Jan 2004 report, Appendix 2, **Figure 7**), which showed that RM1-GFP/CDUPRT cells treated with 5FC above 3 µg/mL showed a marked reduction in proliferation compared with control cells.

In vivo: RM1-GFP/CDUPRT (2.5×10^6) cells were injected subcutaneously (sc) in C57BL/6 immuno-competent male mice and the tumors were harvested when they reached the size 10x10mm. Control mice received RM1-GFP/LacZ cells. The tumors were homogenized in PBS using liquid nitrogen followed by three repeat cycles of freeze (liquid nitrogen)-thawing (37 °C) to complete the cell lysis. The cell debris was removed by centrifugation @ 15,000g for 10 minutes and the homogenates examined for CDUPRT enzymic activity by the HPLC assay as described above (Jan 2005 report, Appendix II, **Figure 5B**). Effective production of 5FU was demonstrated. The sensitivity of the HPLC-based assay was also evaluated and the minimum protein concentration at which the 5FC-5FU conversion was detectable by HPLC was 0.3 mg (Data not shown). This is equivalent to $\sim 2 \times 10^6$ cells in *in vitro* tissue culture. Furthermore, comparison of tumor samples with the cultured cells showed that at 24 h, 6 fold more protein was required from the tumor samples to achieve the same sensitivity. This could be accounted for by the presence of non-tumor tissue in the tumor sample such as stroma and vasculature (Data not shown).

iii) Optimization of viral dose for delivery of CDUPRT-GDEPT

This is in progress.

TASK 1d. Assess ability of optimal doses of CD/UPRT-GDEPT (and control plasmid) injected intraprostatically into RM-1 tumors together with systemic pro-drug (5 fluorocytosine, 5FC) treatment to suppress local prostate and metastatic (lung) tumor development. &

TASK 1e. Examine other tissues for signs of toxicity that might result from escape of the CD/UPRT GDEPT virus from the site of injection.

This requires completion of section c before the studies can be performed. However, we have implanted RM1 cells stably transfected with CDUPRT in the prostate and treated the mice with 5FC to test **proof of principle**.

GDEPT effected by RM1-GFP/CDUPRT cells plus 5FC

We found that the maximum non-toxic dose of prodrug that we could solubilize and use daily was 500mg/kg/day. We first established that RM1-GFP/CDUPRT cells could effect GDEPT *in vivo*.

C57BL/6 mice were implanted in the prostate with 5×10^3 of RM1-GFP/CDUPRT (test) or RM1-GFP/LacZ (control) cells, and 4 days later the prodrug 5FC (or saline, as a control) was administered intraperitoneally (ip) at 500 mg/ kg/day for 14 days. On day 18, mice were sacrificed and their prostate volumes determined using the formula, $V = \pi/6(d_1 \cdot d_2)^{3/2}$, where d_1 and d_2 are diameters at right angles. GDEPT from the use of RM1-GFP/CDUPRT cells plus 5 FC was extremely effective, with almost complete absence of growth in the prostate compared with control cells, or with RM1-GFP/CDUPRT treated with saline (Jan 2005 report, Appendix II, **Figure 6**). Tumors were taken from mice treated with RM1-GFP/CDUPRT with saline (6 mice) or with 5FC (10 mice) and examined histologically after H & E staining by Kim Ow, Oncology Research Centre, POWH. RM1-GFP/CDUPRT tumors from mice given saline all showed >80% viability, with <10% necrosis or haemorrhagic necrosis, whereas those from mice given daily 5FC had no tumor (in 6 cases), or <10% viable tumor (4 cases). In the latter cases, there was >30% necrosis, and >60% haemorrhagic necrosis (Table 1). A comparison of these tumors is shown in the Jan 2005

report, Appendix II, **Figure 7**. Other tissues were taken for histological examination including kidney, lung, spleen, liver, but no abnormal findings were seen in any of the mice whether treated with saline or 5FC. **This indicated that the effects were specific to the tumor, and no other toxicity was observed.** Very little infiltrate was seen in any case.

Table 2: Effects of CDUPRT-GDEPT on prostate histology.

Treatment 4.5 x RM1-GFP/CDUPRT cells injected into the prostate	viability	necrosis	Haemorrhagic necrosis
+ saline ip 15 days (n=6)	>80%	<10%	<10%
+ 5FC ip daily (n=4)	<10%	>30%	>60%
+ 5FC ip daily (n=6)	No	No growth	No growth

Local bystander effect on RM1 growth.

Any local bystander effects from CDUPRT-GDEPT were assessed *in vitro* and *in vivo*.

In vitro: To overcome the problem that different cell lines grew at different rates *in vitro*, we tested conditioned media from RM1-GFP/CDUPRT cells (grown with or without 5FC (at 1mM) or RM1-GFP/LacZ cells (with 5FC) on the growth of RM1 parental cells. The media were harvested 48 h after the addition of 5FC. RM1 parental cells were plated at 5×10^3 cells/well in a 96 well plate, and media conditioned by the different cells were mixed with an equal proportion of fresh medium. Results in Jan 2005 report, Appendix II, **Figure 8A** show that medium collected from RM1-GFP/CDUPRT with 5FC but not RM1-GFP/LacZ cells (with 5FC) prevented the growth of RM1 cells.

In vivo: To assess any local bystander effect of GDEPT treatment on RM1 growth, RM1-GFP/CDUPRT cells and RM1-GFP/LacZ cells were mixed in different proportions for inoculation of mice, that were then treated with 5FC for 14 days at 500 mg/kg/day. The minimal proportion of RM1-GFP/CDUPRT cells required to produce a therapeutic effect were thus established and found to be 20% (Jan 2005 report, Appendix II, **Figure 8B**).

Distant bystander effects of CDUPRT-GDEPT

To determine whether killing of cells in the prostate by CDUPRT-GDEPT would have any effect on the growth of pseudometastases of RM1 cells in the lung, the following experiment was performed.

C57BL/6 mice were injected intraprostatically with 5×10^5 RM1-GFP/CDUPRT or RM1-GFP cells.

Four days later, mice received 2.5×10^5 RM1 cells intravenously, and were treated with 500 mg/kg/day 5FC daily ip for 15 days. Mice were sacrificed on day 19 and their lungs removed, fixed in Bouin's fixative, and the colonies caused by RM1 growth were counted. The results in Jan 2005 report, Appendix II, **Figure 9** show that the number of lung colonies in mice given RM1-GFP/CDUPRT in their prostate was much lower than that in mice given RM1-GFP cells. Thus 50% of mice given RM1-GFP/CDUPRT and treated with 5FC had no lung colonies, whereas those treated with RM1-GFP/CDUPRT and saline, and control mice (that received RM1-GFP cells + 5FC) had no mice in this category. In contrast, >75% mice treated with RM1-GFP/CDUPRT + saline and ~60% of mice that received RM1-GFP cells + 5FC had >100 colonies/lung. This suggests that an immune response, generated by the release of tumor antigens when RM1 cells were killed by GDEPT, may have prevented the growth of lung colonies of RM1 cells injected intravenously. It is also possible that 5FU released by conversion of 5FC by CDUPRT in the

prostate may have had a direct effect on lung colonisation of RM1 cells, and we will perform further experiments to test this possibility.

TASK 1f. Identify using immunohistochemistry, the immune cell types infiltrating the prostate tumors.

We have used parental RM1 tumors not subjected to treatment to perform baseline immune cell determinations by immuno-peroxidase staining with the following MAbs: rat asialoGM1 polyclonal antibody that identifies cells that kill tumors via natural cell mediated cytotoxicity (NCMC); F4/80 that binds to a cell surface glycoprotein on macrophages and their precursors; 53-6.7 that binds CD8 on the surface of CTLs; anti-mouse CD4 found on the surface of helper T cells and anti-mouse CD90 expressed by thymocytes, most peripheral T lymphocytes, some dendritic cells and haemopoietic stem cells, but not by B cells. Some CD90 is also expressed by neuronal cells as well as by epithelial cells and fibroblasts.

RM1 prostate tumors were fixed with 37.5mM periodate-75mM lysine-2% paraformaldehyde, pH 7.4 (PLP), OCT mounted and snap frozen. Frozen sections (5µm) were placed on SuperFrost® Plus slides (Art# 041300, Menzel-Glaser, Germany) and were incubated sequentially with 1.5% H₂O₂ (5 mins), avidin block (10 mins), biotin block (10 mins), 3% bovine serum albumin in Tris (5 mins) to block endogenous peroxidase/biotin and non-specific Mab binding. Tissue was incubated with the primary Mab for 1 h at room temperature and secondary Mab for 15 mins followed by a 15 min incubation with peroxidase-conjugated-streptavidin (1:200 dilution). Otherwise, tissue was incubated with primary Mab 1 h and secondary Mab for 30 mins followed by a 30 min incubation with ABC reagent (Vectastain *Elite* ABC System, Vector Laboratories; 1:200 dilution). Sections were developed with 3,3'-diaminobenzidine (DAB) and counterstained with haematoxylin. Mabs (PharMingen) used in immunoperoxidase staining included: biotin rat anti-mouse CD90 [Thy-1.2] (#01012D; 1:400 dilution); biotin rat anti-mouse CD4 [L3T4] (#553649; 1:400 dilution); biotin rat anti-mouse CD8 [Lyt-2] (#01042A; 1:200 dilution); protein A affinity purified rat anti-macrophage marker (hybridoma clone F4/80; Austyn and Gordon 1980) used at a 1:800 dilution; protein A affinity purified rabbit anti-mouse/rat asialoGM1 polyclonal antibody (Cedarlane International, #486-10001; 1:400 dilution). Secondary reagents used included: biotin conjugated rabbit anti-rat Ig (Vector Laboratories #BA-4001); biotin conjugated goat anti-rabbit Ig (Vector #BO-1000). Stained sections were examined using light microscopy (Leitz Laborlux S; Leica) coupled to a video camera imaging system (Sony Hyper HAD, color CCD-IRIS/RGB, model DXC-151AP) equipped with image analysis software Leica Qwin for PC (Jan 2004 report, Appendix II, Figure 10). From each section, 10 microscopic fields showing the highest positive staining at 40x magnification were selected and the number of positive cells within a fixed 450 x 450 pixel frame (0.1520 mm²) were counted. The average number of positive cells per high power field (HPF) ± SEM for each section was determined. Although few adaptive immune cells identified by the CD4 or CD8 MAbs were observed, some innate immune cells detected by the anti-asialoGM1 (73±43 cells per mm²), F4/80 (100±37 cells per mm²) and anti-CD90 (177±43 cells per mm²) antibodies infiltrated the untreated RM1 tumors (Jan 2004 report, Appendix II, **Figure 10**).

Similar studies have been performed on tumors after injection of RM1-GFP/CDUPRT cells + 5FC treatment or after injection of a mixture of these in different proportions with RM1-GFP cells. In each case, frozen and deparaffinized tissue sections were blocked for endogenous peroxidase (0.3% H₂O₂/H₂O), avidin-biotin (Dako Kit) and non-specific protein binding (3% BSA Ig-free, Sigma). 0.1%Tween₂₀/PBS was used as washing buffer. Intratumoral infiltrating immune cells and microvasculature were detected on freshly obtained tissues that were snap frozen and embedded in Optimal Cutting Temperature compound, OCT (Tissue Tek), sectioned at 5 µm, air-dried then acetone fixed and stained using rat anti-mouse CD4 (BD-Pharmagen) 1:100; rat antimouse CD8a (Ly-

2) (BD-Pharmagen) 1:200; rat antimouse F4/80 (BD-Pharmagen) 1:800 and rabbit antimouse AsialoGM1 (Wako) 1:400, or rat antimouse 1:300 CD31 (BD-Pharmagen), respectively by incubating for 45 mins at room temperature followed by incubation with secondary antibodies, anti-rat (1:200) and rabbit (1:200) and the ABC complex for 15 mins. The standard avidin-biotin-peroxidase complex (ABC) detection system and the diaminobenzidine (DAB) as the chromagen and Harris haematoxylin as counterstain were used. In addition, tissues were fixed in 10% neutral buffered formalin (Amber Scientific) and paraffin-embedded after vacuum infiltration processing in Tissue Tek-VIP (Sakura) for immunostaining for proliferating tumor cells or apoptotic cells. Paraffin sections were dewaxed in Histochoice (Ambresco), rehydrated through graded series of ethanol and washed in PBS before blocking. Proliferating tumor cells were assessed in sections immunostained overnight with rat antimouse Ki67 (Dako) 1:10 at 4⁰C after pretreatment with antigen retrieval solution, performed by microwave irradiation in 0.01 M citrate buffer pH6, according to the suppliers' protocols for Ki-67 antibody and the TUNEL assay. The secondary-linked antibody, anti-rat (1:200) and the ABC complex (1:200) were incubated for 30 mins. Apoptotic cells were detected using the TUNEL assay kit (Roche), also after antigen retrieval.

Scoring of positive stained cells was performed by light microscopy as follows. From each immunostained section, areas containing high numbers of positive cells were initially identified by systematically scanning under x100 low power magnification. Individual positive cells observed in the ten fields under higher magnification x400 (0.15 mm square), showing the highest positive staining were manually counted and the average number of positive cells per high power field (HPF \pm SEM) was determined. Because of the high number of positive apoptotic cells, a higher magnification of x620 was used and cells were counted in an area representing 25% of the high power field. To minimise false positive staining resulting from extensive DNA fragmentation in late stages of necrosis, the morphology of stained cells were carefully examined. We had also observed that positive brown colour nuclear stained apoptotic tumours cells were morphologically distinguishable from the degenerative tumours (similarly stained brown in color), such as pyknotic necrosis with small condensed chromatin and coagulative necrosis consisting of ghost-like cells

Vascular hot spots were not observed in the present study. Vascular analysis of the entire tumor section was carried out. Any brown staining endothelial cell or endothelial cell cluster separated from adjacent microvessels was included into the counting procedure and counted as one microvessel. Neither vessel lumens nor red blood cells were used to define a microvessel (Vermeulen et al 1996). Infrequent CD31-positive macrophages and plasma cells were excluded from the counting procedure.

The data obtained are summarized in Table 3. Increasing numbers of CD4 positive infiltrating immune cells were detected in all three RM1-GFP/CDPURT+5FC treatment groups (B,C,D) as compared to the RM1-GFP+5FC control group (A). CD4 cells recruitment was the most significant in the RM1-GFP/CDPURT+5FC tumor in the treatment group B when compared with the 20% and 10% mixed cells tumors (see Jan report 2005, Appendix II, **Figure 10A, B**). In contrast, staining for CD8a positive cells was minimal. Increasing numbers of F4/80-positive and Asialo GM1+ infiltrating immune cells were detected in tumors of the three RM1-GFP/CDPURT+5FC treatment groups (B,C,D) as compared with the control group (A) (Table 3); representative sections from treated tumors are shown in Jan report 2005, Appendix II, **Figure 10C** and **Figure 10D** respectively.

Of interest was the finding that CDUPRT-GDEPT + 5FC caused a reduction in the vascularity of the tumors (Table 3, Jan report 2005, Appendix II, Figure 11A). No positive Ki67 tumor cells were detected in the treated and control tumors (except one tumor from the RM1-GFP/CDURPT+5FC treatment which shows a 25% Ki 67 proliferating tumor cells (Jan report 2005, Appendix II Figure 11B) but a marked increase in apoptosis was seen following CDUPRT-GDEPT (Table 3, Jan report 2005, Appendix II, Figures 11C and 11D).

Table 3. Immunohistochemical analysis of infiltrating immune cells, endothelial cells (vasculature) and apoptotic tumor cells in RM1-GFP/CDURPT+5FC and control tumors

Treatment	Infiltrating immune cells						Vasculature (endothelial cells)		Apoptotic tumor cells	
	CD4		CD8a		F4/80		AsialoGM1		CD31	
	Mean*	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
(A) RM1-GFP+5FC (Control)	4.55	0.39	1.26	0.40	10.85	1.90	13.05	1.10	18.03	2.89
(B) RM1-GFP/CDURPT+5FC+saline	22.04	7.51	1.70	1.70	22.00	4.71	20.27	3.94	5.67	0.35
(C) 20%RM1-GFP/CDURPT+5FC mixed with 80%RM1-GFP	9.40	3.46	1.73	0.90	16.23	2.38	16.20	2.76	8.33	0.13
(D) 10%RM1-GFP/CDURPT+5FC mixed with 90% RM1-GFP	9.43	3.36	4.25	1.27	20.53	0.76	14.93	1.82	13.82	2.09
									47.03	3.74
									84.25	0.75
									72.97	2.17
									65.30	0.62

* Mean number of positively stained cells/high power field from a count of 10 fields. SEM: Standard error of the mean

We have demonstrated proof of principle that CDUPRT-GDEPT + 5FC kills prostate cancer cells when given into the prostate, and also inhibits the growth of pseudometastases in the lung. Immune cells infiltrating the primary tumor include CD4 and CD31 positive T cells, F480 positive macrophages and AsialoGM1+ NK cells. In addition, there was a decrease in the vasculature to the tumor, and an increase in apoptosis as measured by TUNEL (Table 1).

As we now have the lentiviruses, an assay for CDUPRT activity after delivery in vivo, and a usable dose for the 5FC, we are in an excellent position to compare the data obtained using CDUPRT-RM1 transfected cells with viral delivery of CDUPRT.

Task 2.

pCytokine work: Assess the ability of lipid-enhanced delivery of an murine IL-12 or IL-18 expressing plasmids (pCytokine) to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 12-22)

TASK 2a: Prepare pCytokine constructs

Some of this work was initiated and reported in our January 2004 report. Plasmids expressing the murine IL-12 or murine IL-18 cytokines were prepared as pVITRO2-GFP-Cytokine (murine IL-12 and IL-18) constructs (see Task 2, sections (i), characterized and in the case of the mIL18 construct, sequenced (Jan 2004 report, Appendix III). These were called pVITRO2-GFP/mIL12 (Jan 2004 report, Appendix II, **Figure 11**) and pVITRO2-GFP/mIL18 (Jan 2004 report, Appendix II, **figure 14**).

To ensure that the RM1 prostate cancer cell lines would not be stimulated by exposure to these cytokines, they were tested for the presence of surface IL12 receptor (IL12R) and IL18 receptor (IL18R) by FACS analysis using suitable antibodies as described in Jan 2004 report, Task 2, sections (i) and (ii) and Jan 2004 report, Appendix II, **Figures 12 and 15**. The RM-1 cells did not express either IL12R or IL18R.

TASK 2b: Determine dose of pCytosine-construct plasmid DNA (0.1-1.0 µg) which, when formulated with lipid and control virus leads to detectable expression of cytokine in orthotopically implanted RM-1 tumors by:

(i) Harvesting tumor cells, culturing and monitoring cytokine production by Western blot.

Whilst the lentiviral constructs expressing the cytokines were being prepared, we generated stable transfectants of RM1-GFP/IL12 and RM1-GFP/IL18 under hygromycin B selection (800 µg/mL) as described in the January 2004 report, under Task 2. Direct sequencing confirmed the appropriateness of the plasmid inserts. The same control cells as for RM1-GFP/CDUPRT, stably transfected to express GFP alone or GFP/LacZ, and known as RM1-GFP or RM1-GFP/LacZ (controls) respectively, were used. Again, GFP was used as a marker to follow the cells *in vivo*, and to sort high expressers as described previously (see Jan 2004 report, Appendix II, **Figures 5, 6, 13**).

Gene expression by RM1-GFP/mIL12 and RM1-GFP/mIL18 cells:

1. Protein detection assay: mIL12 and mIL18 expression by RM1-GFP/mIL12 or RM1-GFP/mIL18 cells was examined by ELISA (BD Pharmingen) but the assay was not successful for cell supernatants. As an alternative, a western blot based assay is currently being optimized. The preliminary data obtained thus far indicate the presence of secreted cytokines in both cell lines (data not shown).

2. Functional assay: This was developed to assess the functionality of the cytokines produced by these cell lines. To minimize the use of animals as spleen cell donors for the traditional functional assays for IL12 and IL18, an assay based on the use of murine CTLL2 cells was developed. The rationale for the assay was based on the following two facts:

1. At least 60% of CTLL2 cell population has mIL12 receptors as shown by the data obtained from FACS-based analysis of immunostaining of CTLL2 cells with mIL12 receptor antibody. (see January 2004 report, Appendix II, figure 12).
2. IL12 and IL18 synergize with each other by upregulating each other's receptors. IL-12 can upregulate the production of the IL-18 receptor α (IL18R α) chain on Th1 cells (Ahn *et al.*, 1997) whereas IL-18 has been shown to upregulate the IL12R β 2 chain on Th1 cells (Chang *et al.*, 2000). This shared upregulation of receptors provides a positive feedback mechanism allowing these cytokines to act synergistically.

Method: CTLL2 cells (obtained from Dr Peter Williamson's laboratory from S Schibeci at Millennium Institute at Westmead Hospital, Sydney) are derived from C57BL/6 mice and grow indefinitely in culture in the presence of 20u/mL of mIL2 (Roche). In general, 5×10^3 cells were seeded in a 96-well plate and at the relevant times post treatment, the proliferation was measured by 4 h incubation of the cells in WST1 (10 μ L/100 μ L, Scientifix). As the assays were being developed to examine the proliferative effects of mIL12 and mIL18 on CTLL2 cells, the normal doses of mIL2 (20 u/mL, Roche, USA) would have masked the effects of other cytokines. The aim of the first set of experiments was to determine a suboptimal concentration of mIL2 that would allow the cells to survive but not to proliferate. Cells were incubated with different doses of mIL2 (50-0 units/ml; January 2005 report, Appendix II, **Figure 12**) and analysed for proliferation at 24, 48 and 72 h. *These data suggested that at 2u/L these cells were able to survive until 72h without proliferating.* From this point on all assays were done using assay media containing mIL2 at 2 u/mL. Furthermore, the integrity of the cells was determined by the proliferative response of CTLL2 cells to mIL2 in all experiments.

Experiment 1: *CTLL2 cells respond to the presence of recombinant mIL12 and mIL18 in the growth medium.*

Cells were incubated with recombinant mIL12 (R&D systems, USA) or mIL18 (Medical and Biological Laboratories Co., LTD, Japan) (ranging from 0-100 ng/mL) for 72 h. The cells showed a clear proliferative response to both cytokines. This was enhanced by 3.2 fold when treated with mIL12 (100ng/ml, January 2005 report, Appendix II, **Figure 13A**) and by 3.7 fold when treated with mIL18 (0.1 ng/mL; January 2005 report, Appendix I, **Figure 13B**).

Experiment 2: *CTLL2 cells respond to the supernatants from RM1-GFPmIL12 and RM1-GFPmIL18.*

Cells were incubated with supernatants at concentrations ranging from 100% to 0% from RM1-GFPmIL12 and RM1-GFPmIL18 cells, respectively. Since we currently have no estimate of the quantities of the cytokines produced by these cell lines, a % of the supernatants was applied. As a control, supernatant from RM1-GFP cells was used in both cases. Measurement of absorbance (at 450nm) at 72 h post treatment clearly showed that proliferation was enhanced 4.7-6.9 fold by RM1-GFPmIL12 supernatant compared to the RM1-GFP controls (January 2005 report, Appendix II, **Figure 14A**). However, even though the proliferative response of CTLL2 cells to supernatants from RM1GFPmIL18 was obvious (~2.5 fold at 12.5 and 6.25%; January 2005 report, Appendix II, **Figure 14B**), it was not as significant as that observed with mIL12 containing supernatants.

Experiment 3: CTLL2 proliferation is further enhanced when treated with both mIL12 and mIL18 in tandem.

As the proliferative response of the CTLL2 cells to mIL18 was not as strong as that observed with mIL12, this experiment was designed to enhance the proliferative effects of the two cytokines by using them in combination. *The hypothesis was that initial incubation with mIL12 would upregulate the mIL18 receptors in CTLL2 cells and this would lead to an enhanced response of the CTLL2 cells to mIL18 as a follow up treatment (refer above for the relevant references).* Cells were treated with a range of concentrations of mIL12 (0-100ng/mL) and after 24 h incubation mIL18 was added to the cultures at varying concentrations (0-100ng/mL). Cells treated with both cytokines together showed an enhanced proliferation compared with either alone. Specifically, this enhancement was significant at higher concentrations of mIL12 (10 and 100 ng/mL) (January 2005 report, Appendix II, **Figure 15**). Maximal effects were observed when mIL12 at 10ng/ml was combined with mIL18 at 1, 10 or 100ng/ml. Significantly, the enhancement was ~10 fold with mIL12 at 10 ng/mL and mIL18 at 100 ng/mL.

The experiments described above have shown that:

1. CTLL2 cells respond to both mIL12 and mIL18.
2. RM1-GFPmIL12 and RM1-GFPmIL18 secrete functional cytokines.
3. The proliferative response of the CTLL2 cells is further enhanced when the two cytokines are used in combination.

To further support these observations we plan to show conclusively that these effects are mediated by the cytokines by using the relevant neutralising antibodies to the two cytokines.

Note: This functional assay has not only facilitated the assay of the two cytokines without the use of animals in this laboratory, but also the data generated are sufficient to submit as a technological note to Journal of Immunological Techniques. This paper is in preparation.

(ii) TASK 2b: Measuring biological activity of secreted cytokine using a cytotoxic lymphocyte (CTL) bioassay.&

(iii) TASK 2b: Measuring cytokine mRNA production by RT-PCR.

This work has not been done. Instead, we have examined the effects of mIL12 and mIL18 on *in vivo* growth of RM1 cells by implanting the stably transfected RM1-GFP/mIL12 or RM1-GFP/mIL18 cells, as described below.

In vivo studies using RM1-GPF/mIL12 cells: In order to investigate the effects that IL-12 expression alone has on growth and to determine a suitable cell number for future RM1-GFP/IL12 implantations, C57BL/6 male mice were initially injected sub-cutaneously (sc) with either RM1-GFP/IL12 cells or RM1-GFP/LacZ control cells at 1.5×10^4 , 1.5×10^5 or 1.5×10^6 cells/mouse. Tumor growth was measured on days 4, 7 and 14. Tumor samples were harvested on day 14 and stored embedded in OCT media at -80°C or were formalin fixed and paraffin embedded for storage at room temperature.

Table 4 Take rate of subcutaneous RM1-GFP/mIL12 and RM1-GFP/LacZ cells in C57BL/6 mice

Cells Implanted	Day 7 Take Rate	Day 14 Take Rate
1.5×10^4 RM1-GFP/LacZ	0%	33%
1.5×10^5 RM1-GFP/LacZ	100%	100%
1.5×10^6 RM1-GFP/LacZ	100%	100%
1.5×10^4 RM1-GFP/IL12	0%	40%
1.5×10^5 RM1-GFP/IL12	66%	66%
1.5×10^6 RM1-GFP/IL12	0%	60%

At cell numbers of $\geq 1.5 \times 10^5$ cells, RM1-GFP/IL12 cells had both a lower take rate (60-66% compared with 100%) (Table 1) and slower growth than equivalent numbers of RM1-GFP/LacZ cells treated similarly (January 2005 report, Appendix II, **Figure 16A**). Moreover increasing the number of cells injected caused slower growth (lower average tumor volume, January 2005 report, Appendix II, **Figure 16A**) and a lower take rate (1.5×10^6 RM1-GFP/IL12 cells 60% take rate vs. 1.5×10^5 RM1-GFP/IL12 cells, 66% take rate, Table 4). Thus mice injected with 1.5×10^5 cells showed no detectable tumors by Day 7, in contrast to those receiving 1.5×10^5 RM1-GFP/mIL12 cells, or mice injected with 1.5×10^5 cells and 1.5×10^6 cells RM1-GFP/LacZ cells. These data suggest that the mIL12 was biologically active and secreted, inhibiting tumor take and growth. Hence, formalin fixed tumor sections were H&E stained and analysed by light microscopy to assess immune cell infiltration of these tumors (See January 2004 report, Appendix II, **Figure 13A**). Examination of these sections showed that the control tumors (RM1-GFPLacZ) in general, showed no evidence of immune cell infiltration and resulting necrotic areas. In contrast, the RM1-GFPmIL12 tumors were mostly necrotic and heavily infiltrated with immune cells. The nature of the infiltrating cells will be further analyzed by immunostaining with specific antibodies against immune cell populations. *These results are promising given our plan to combine immunotherapy and GDEPT in the proposed study.*

A second experiment confirmed that the growth of RM1-GFP/IL12 cells was slow and achieved a smaller volume (note different scales on the X axis, January 2005 report, Appendix II, **Figure 16B**) than parental RM1 or control RM1-GFP cells. Sections of the tumors taken at sacrifice were stained with H&E to assess immune cell infiltration. When examined histologically sections from sc RM1-GFP/IL12 tumors showed more extensive necrosis and immune cell infiltration than those from RM1-GFP/LacZ s (See Jan 2004 report, **Figure 13A**)

Similarly, when RM1-GFP/IL18 cells (3×10^5) were inoculated sc into C57BL/6 mice, the growth of the cells was extremely slow and the tumor size achieved was much smaller compared with that of parental RM1 or control RM1-GFP cells (January 2005 report, Appendix II, **Figure 16C**; note differences in X axes). In rare cases, rapid growth of a given tumor suddenly occurred suggesting outgrowth of a modified clone of cells.

When RM1-GFP/IL12 or RM1-GFP/IL18 cells were injected into the prostate of C57BL/6 mice, the volume of tumor growth appeared to be related to cell number (January 2005 report, Appendix II, **Figure 17**), but histological examination indicated that tumors arising after injection of larger cell numbers showed decreased viability, with increased haemorrhagic necrosis than those from injection of lower cell numbers (Table 2). The following parameters were assessed: extent of vascularisation, % Viable tumor; % Tumor necrosis (assessed as pyknotic necrosis (with dense

chromatin) at the margin of viable tumors or coagulative necrosis (ghost-like cells) further away from the blood vessels, and % haemorrhagic necrosis.

Inhibition of tumor growth by RM1-GFP/mIL12 and RM1-GFP/mIL18 cells compared with control cell lines after either sc or intraprostate inoculation clearly indicate cytokine-transfected RM1 cells are expressing biologically active mIL12 and mIL18, respectively when implanted *in vivo*.

(iv) TASK 2b: Identify using immunohistochemistry the immune cell types infiltrating cytokine secreting tumors

In addition, frozen sections of the orthotopic tumors arising from injection of RM1-GFP/IL12 cells were examined for infiltrating mouse immune cells, by staining with Rabbit-anti Asialo GM1 (*Wako Pure Chemical Industries* C/N 986-10001, diluted 1/00), that detect NK cells, CD4, CD8 (BD Pharmingen, C/N 550281, diluted 1/200) and CD90 (CD90.2 (Thy 1.2) BD Pharmingen C/N 550543, diluted 1:400) that detect T cells, and F4/80 (BD Pharmingen, C/N 552958, diluted 1/800) that detects macrophages. Anti-rat and anti-rabbit antibodies were used as a secondary-

Table 5: Histological changes in tumors arising in the prostate after injection of RM1-GFP/IL12 or RM1-GFP/IL18 cells.

<u>Treatment group no</u>	<u>Mouse no</u>	Tumor cells injected	Prostate tumor diameters (mm)	Histology			
				Vascularity	% Haemorrhagic necrosis	% Tumor Necrosis	% Viable Tumor
RM1-IL12 #1	168.1	4.5×10^3	10x7	High	>2	>5	<80
	168.2	4.5×10^3	10x10	High	>2	>10	<80
RM1-IL12 #2	189.2	4.5×10^4	6x4	High	ND	>5	<80
	190.1	4.5×10^4	12x12	High	>10	>35	<50
	190.3	4.5×10^4	8x7	ND	>20	>35	<40
RM1-IL12 #3	187.1	4.5×10^5	13x10	ND	>20	>30	<40
	187.3	4.5×10^5	6x4	ND	>40	>40	<10
	188.2	4.5×10^5	15x15	ND	>40	>40	<20
RM1-IL18	182.1	$4.5 \times 10^?$	10x5	Moderate	>20	>30	<35

linked antibodies at the optimal concentrations as shown: Biotinylated rabbit anti-rat: 1:200 (for CD8a, CD90 and F4/80), Vector Lab C/N I0813 and Biotinylated goat anti-rabbit: 1:1000 (for Asialo GM1), Vector Lab, C/N K0323.

Intraprostate tumors arising from injection of control cells, RM1 or RM1-GFP contained no CD8 cells, or F4/80 positive cells, but there were few asialo-GM1 positive cells, and some CD90 positive cells around blood vessels. In contrast tumors from RM1-GFP/IL12 cells showed an increase in infiltrating immune cells, in particular NK (Asialo-GM1 positive cells, seen in clusters), both CD8 positive (few) and CD90 positive (especially around blood vessels and in haemorrhagic necrotic areas) and some F4/80 positive cells (see January 2005 report, Appendix II, **Figure 18**).

In our next experiments, we will complete Task 2, by investigating whether anti-prostate cancer activity can be identified in infiltrating lymphocytes within the prostate after CDUPRT-GDEPT, or after injection of RM1-GFP/IL12 or IL18 cells, and examine the effects of immune cell depletion on these activities. We will then be in a position to begin Task 3.

The following studies have not yet been undertaken

TASK 2c. Determine impact of administering 5-FC prodrug on immune cell recruitment into tumors following injection of cytokine gene plasmid/lipid/control virus administration &

TASK 2d. Determine the persistence of cytokine production by transfected tumors.

TASK 2e. Compare ability of transfected pCytokines (optimal dose complexed with lipid and control virus) to suppress orthotopic and metastatic RM-1 prostate tumor growth.

TASK 2f. Choose optimal cytokine gene system on basis of maximum suppression of tumor growth obtained.

TASK 3. Combination therapy: Assess the ability of delivery of a combined virus borne GDEPT and lipid delivered plasmid-borne cytokine gene therapy to suppress orthotopic and metastatic prostate cancer in RM-1 model and in TRAMP mice carrying sc TRAMP-C1 grafts. (Months 22-33)

- a. Determine whether pCytokine-enhanced immune activity affects GDEPT.
- b. Determine the effects of injecting lenti virus expressed GDEPT and pCytokine intraprostatically (using optimal doses of each component as revealed by Tasks 1A and 1B) on orthotopic tumor growth and metastases.

TASK 4. Tissue slice work: Assess the ability of OAV encoding green fluorescence protein (GFP) under a prostate directed promoter from Dr Paul Rennie, Vancouver to express GFP in human tissue slices. (Months 24-33)

Task 5. Collate data, prepare reports and manuscripts (Months 33-36)

KEY RESEARCH ACCOMPLISHMENTS:

- Established stably transfected murine prostate cancer lines from RM1 that express the transgenes and the reporter gene, green fluorescence protein: RM1-GFP/CDUPRT cell line; RM1-GFP/mIL12 and RM1-GFP/mIL18 cell lines.
- Established and tested assay systems to measure expression of the transgene, CDUPRT *in vitro* and *in vivo*.
- Shown that CDUPRT-GDEPT + 5FC is associated with a *local bystander effect*
- Shown that CDUPRT-GDEPT + 5FC given into the prostate is also associated with a *distant bystander effect*, resulting in a reduction of the growth of pseudometastases in the lungs of RM1 cells, and with immune cell infiltration in the prostate
- Shown that CDUPRT-GDEPT +5FC effect is associated with infiltration of the tumors with immune cells, especially, macrophages, NK cells and CD4+ cells. Also the treated tumors show greater levels of necrosis and apoptosis, disrupted vasculature and enhanced proliferation in comparison to control tumors. This is encouraging as it suggests further augmentation of the immune responses when CDUPRT-GDEPT is used in conjunction with the cytokine gene therapy.
- Established that RM1-GFP/mIL12 and RM1-GFP/mIL18 secrete biologically active mIL12 and mIL18 respectively by *in vitro* assay.
- Shown that RM1-GFP/mIL12 and RM1-GFP/mIL18 inhibit tumor take rate and tumor growth after implantation either subcutaneously or in the prostate of C57BL/6 mice.

REPORTABLE OUTCOMES:

- Establishment of new cell lines derived from RM1: RM1-GFP/CDUPRT; RM1-GFP/mIL12; RM1-GFP/mIL18.
- Establishment of new androgen-refractory TRAMP cell lines. (TOW prize poster) (paper in preparation)
- Determined new methodology for testing the effects of mIL12 and mIL18 and a combination of these using CTLL2 cells.
- Paper on local and distant bystander effects of CDUPRT-GDEPT and 5FC for PC in preparation.
- Submitted an abstract for AACR conference, April, 2005.
- Dr Rosetta Martiniello-Wilks has been appointed as a Senior Hospital Scientist at Royal Prince Alfred Hospital to set up a GLP facility for Gene Therapy trials. She was the successful candidate for this position because she was a DOD Trainee-fellow.

CONCLUSIONS:

- At this stage of the work, we have 3 papers in preparation and two abstracts.
- We have proof of principle that CDUPRT-GDEPT + 5FC is an effective therapy against RM1 tumor cells grown in the prostate of C57BL/6 mice. This treatment is associated with a local bystander effect, and stimulates a distant bystander effect as evidenced by inhibition of pseudo-metastasis formation in the lungs after intravenous injection of RM1 cells.
- We have clearly shown using CTLL2 cells that mIL12 and mIL18 together exhibit increased effects over either alone in increasing cellular proliferation.
- We have shown that RM1-GFP/mIL12 and RM1-GFP/mIL18 cell lines express biologically active cytokines that inhibit tumor take and tumor growth after implantation under the skin or in the prostate.

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APPENDICES:

Appendix I contains new Statement of Work approved by DOD.

Appendix II contains figures 1-18.

Appendix III Publications and papers in preparation

APPENDIX I:

STATEMENT OF WORK

Task 1. GDEPT alone: Assess the ability of delivery of a lentivirus expressing GDEPT (based on the fusion gene, cytosine deaminase/uracil phosphoribosyltransferase (**CD/UPRT**) to suppress orthotopic and metastatic prostate cancer in RM-1 model. (Months 1-12).

- a. Prepare recombinant lentivirus (using vector from Invitrogen).
- b. Establish conditions for implanting TRAMP-C1 cells sc in TRAMP mice.
- c. Optimize dose of virus needed to establish CD/UPRT GDEPT in orthotopically implanted RM-1 tumors when formulated with lipid and control plasmid.
- d. Assess ability of optimal doses of CD/UPRT-GDEPT (and control plasmid) injected intraprostatically into RM-1 tumors together with systemic pro-drug (**5 fluorocytosine, 5FC**) treatment to suppress local prostate and metastatic (lung) tumor development.
- d. Examine other tissues for signs of toxicity that might result from escape of the CD/UPRT GDEPT virus from the site of injection.
- e. Identify using immunohistochemistry, the immune cell types infiltrating the prostate tumors.

Task 2. pCytokine work: Assess the ability of lipid-based delivery of an , IL-12 or **IL-18** expressing plasmid (pCytokine) or a **combination of both** to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 12-22)

- a. Prepare pCytosine constructs.
- b. Determine dose of pCytosine-construct plasmid DNA (0.1-1.0 µg) which, when formulated with lipid and control virus leads to detectable expression of cytokine in orthotopically implanted RM-1 tumors by:
 - (v) Harvesting tumor cells, culturing and monitoring cytokine production by Western blot.
 - (vi) Measuring biological activity of secreted cytokine using a cytotoxic lymphocyte (CTL) bioassay.
 - (vii) Measuring cytokine mRNA production by RT-PCR.
 - (viii) Identify using immunohistochemistry the immune cell types infiltrating cytokine secreting tumors
- c. Determine impact of administering 5FC prodrug on immune cell recruitment into tumors following injection of cytokine gene plasmid/lipid/control virus administration
- d. Determine the persistence of cytokine production by transfected tumors.
- e. Compare ability of transfected pCytokines (optimal dose complexed with lipid and control virus) to suppress orthotopic and metastatic RM-1 prostate tumor growth.
- f. Choose optimal cytokine gene system on basis of maximum suppression of tumor growth obtained.

Task 3. Combination therapy: Assess the ability of delivery of a combined virus borne GDEPT and lipid delivered plasmid-borne cytokine gene therapy to suppress orthotopic and metastatic prostate cancer in RM-1 model and in TRAMP mice carrying sc TRAMP-C1 grafts. (Months 22-33)

- c. Determine whether pCytokine-enhanced immune activity affects GDEPT.
- d. Determine the effects of injecting lenti virus expressed GDEPT and pCytokine intraprostatically (using optimal doses of each component as revealed by Tasks 1A and 1B) on orthotopic tumor growth and metastases.

Task 4. Tissue slice work: Assess the ability of OAV encoding green fluorescence protein (GFP) under a prostate directed promoter from Dr Paul Rennie, Vancouver to express GFP in human tissue slices. (Months 24-33)

Task 5. Collate data, prepare reports and manuscripts (Months 33-36)

APPENDIX II

Figures 1-18

Figure 1: Construction of pLent.GFP and pLent.CDUPRT by *in vitro* recombination between pENTR.CDUPRT or pENTR.GFP and plenti6/V6-DEST vectors.

Figure 2: Functional testing and optimization of Lent.GFP production in 293FT cells.

Figure 3: Functional testing of Lent.CDUPRT on 293A cells. Figure 4: Growth of TRAMP C1/C2-derived androgen independent cell lines in TRAMP null mice.

Figure 5: Characterization of RM1-GFP/CDUPRT cells. A. *in vitro*; B *in vivo*

Figure 6: GDEPT effected by RM1CDUPRT cells *in vivo*.

Figure 7: Effects of CDUPRT-GDEPT on histology of RM1 tumors

Figure 8: (A) *In vitro* evaluation of the local bystander effect of CDUPRT-GDEPT; (B) Local bystander effect of CDUPRT-GDEPT *in vivo*.

Figure 9: Distant bystander effect of CDUPRT-GDEPT *in vivo*.

Figure 10: Intratumoral infiltrating immune cells following CDUPRT-GDEPT

Figure 11: Effects of CDUPRT-GDEPT on angiogenesis, tumor cell proliferation and tumor cell apoptosis

Figure 12: Proliferation of CTLL2 cells in response to mIL2 at 72 hr post-treatment.

Figure 13: CTLL2 proliferation in response to recombinant mIL12 (A) and mIL18 (B).

Figure 14: CTLL2 proliferation in response to stimulation by supernatants from

RM1-GFPmIL12 (A) and RM1-GFPmIL18 cells (B). Supernatants from

RM1-GFP cells were used as the controls.

Figure 15: CTLL2 proliferation in response to combination of recombinant mIL12 and mIL18. Cells were incubated with mIL12 for 24 hrs followed by addition of mIL18, and then analysed for proliferation 48 hrs later. The data are normalized for no addition of mIL12 or mIL18

Figure 16: Growth of RM1-GFP/IL12 or RM1-GFP/IL18 cells sc in C57BL/6 mice

Figure 17: Relative growth of RM1-GFP/IL12 and RM1-GFP/IL18 cells in the prostate of C57BL/6 mice after orthotopic implantation. RM1-GFPmIL12 (A) and RM1-GFPmIL18 cells (B).

Supernatants from RM1-GFP cells were used as the controls.

Figure 18: Photomicrographs of immune reactive cell infiltration (x10) in RM1 tumors expressing IL12.

Figure 1: Construction of pLent.GFP and pLent.CDUPRT by *in vitro* recombination between pENTR.CDUPRT or pENTR.GFP and pLenti6/V6-DEST vectors.

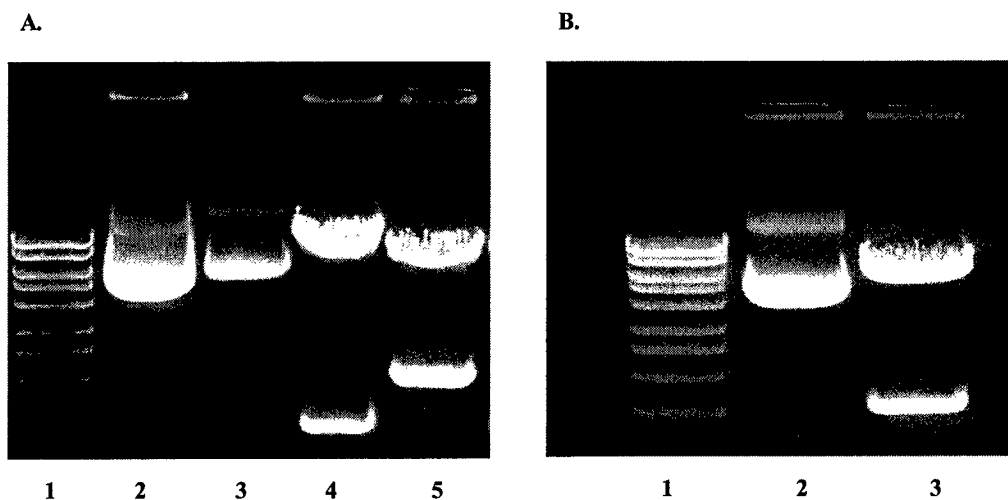


Figure 1: Construction of pLent.GFP and pLent.CDUPRT by *In vitro* recombination between pENTR.CDUPRT or pENTR.GFP and plenti6/V6-DEST vectors: Agarose gel image shows the positive clones for pLent.CDUPRT (panel A) and pLent.GFP (panel B) constructs. Panel A. Clones for pLent.CDUPRT were screened using restriction enzymes *KPN1* (lane 4 fragment size; 7703 and 1342bp) and *ECOR1* (lane5, Fragment size; 6604 and 2007bp). Uncut DNA was electrophoresed in lanes 2 and 3). Panel B. *KPN1* digest based screening for pLent.GFP clone (lane 3, Fragment size; 6000 and 1700 bp). Uncut DNA is represented in lane 2. Lane 1 in both panels represents high range markers (fermentas).

Figure 2: Functional testing and optimization of Lent-GFP production in 293FT cells

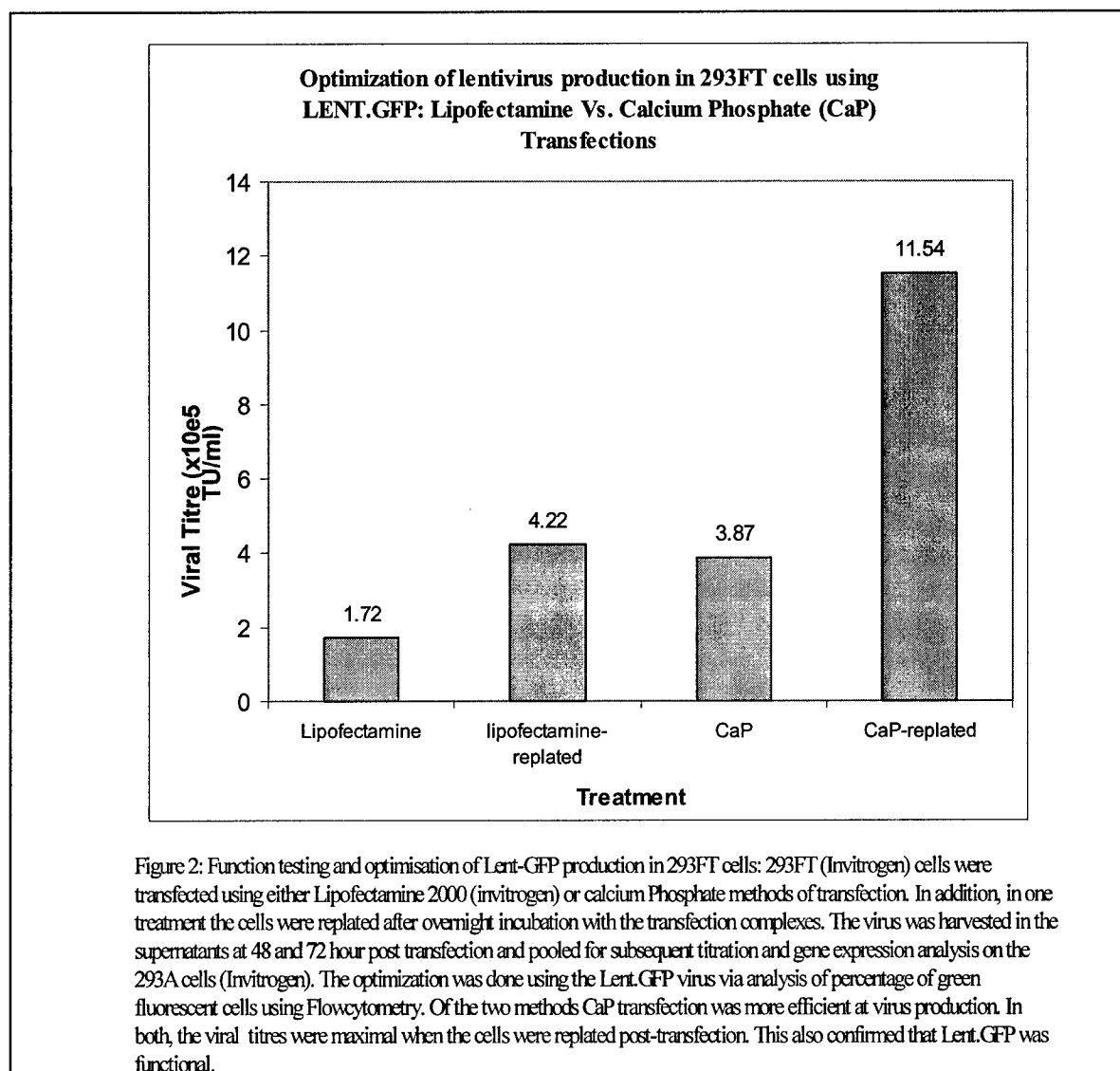


Figure3: Functional testing of Lent.CDUPRT on 293A cells.

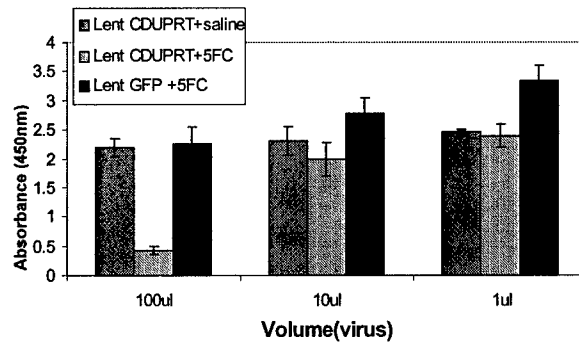


Figure 3: Functional test of Lent.CDUPRT on 293A cells.

Lent.CDUPRT supernatants from 293FT cells were used to transduce 293A cells at three different doses for 48 h and then the cells were treated with 5-fluorocytosine at 1mM concentration. 72 h post treatment the cells were analysed for proliferation using WST1 reagent and absorbance was measured at 450nm. Lent.CDUPRT transduced 293 cells in the absence of 5FC and 293 cells transduced with the control virus, Lent.GFP, in the presence of 5FC, served as the controls for this experiment.

Figure 4: Growth of TRAMP C1/C2-derived androgen independent cell lines in TRAMP null mice.

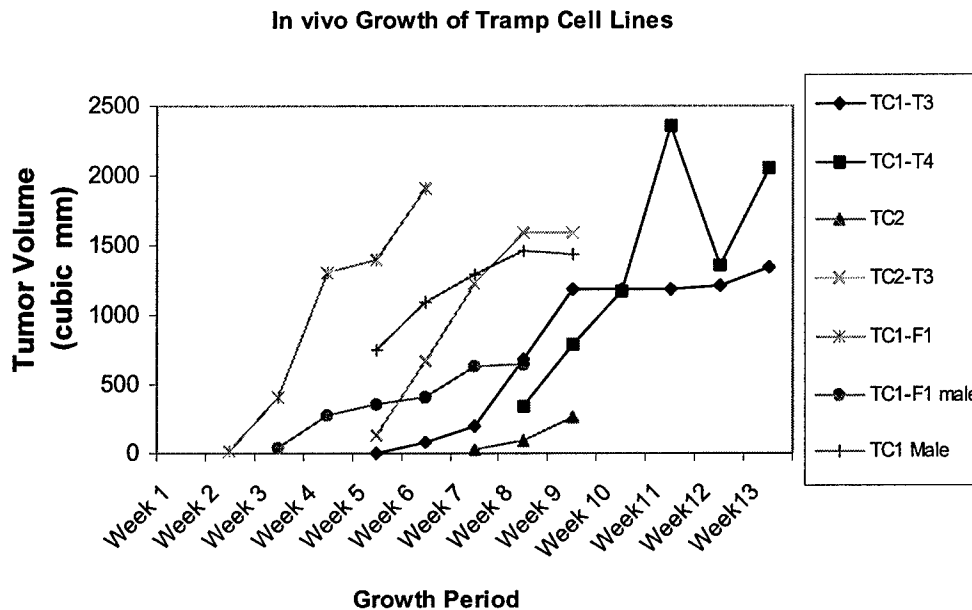


Figure 4: Growth of TRAMP C1/C2-derived androgen independent cell lines in TRAMP null mice: TRAMPC1/C2(parental) cell lines were grown in TRAMP null male mice. Derived androgen-independent cell lines (TC1-F1(grown in males and females), TC2-T3 (grown in female mice), TC1-T4 (grown in female mice), TC1-T3 (grown in female mice) were injected subcutaneously in TRAMP null male or female mice. The growth of the tumors was monitored weekly.

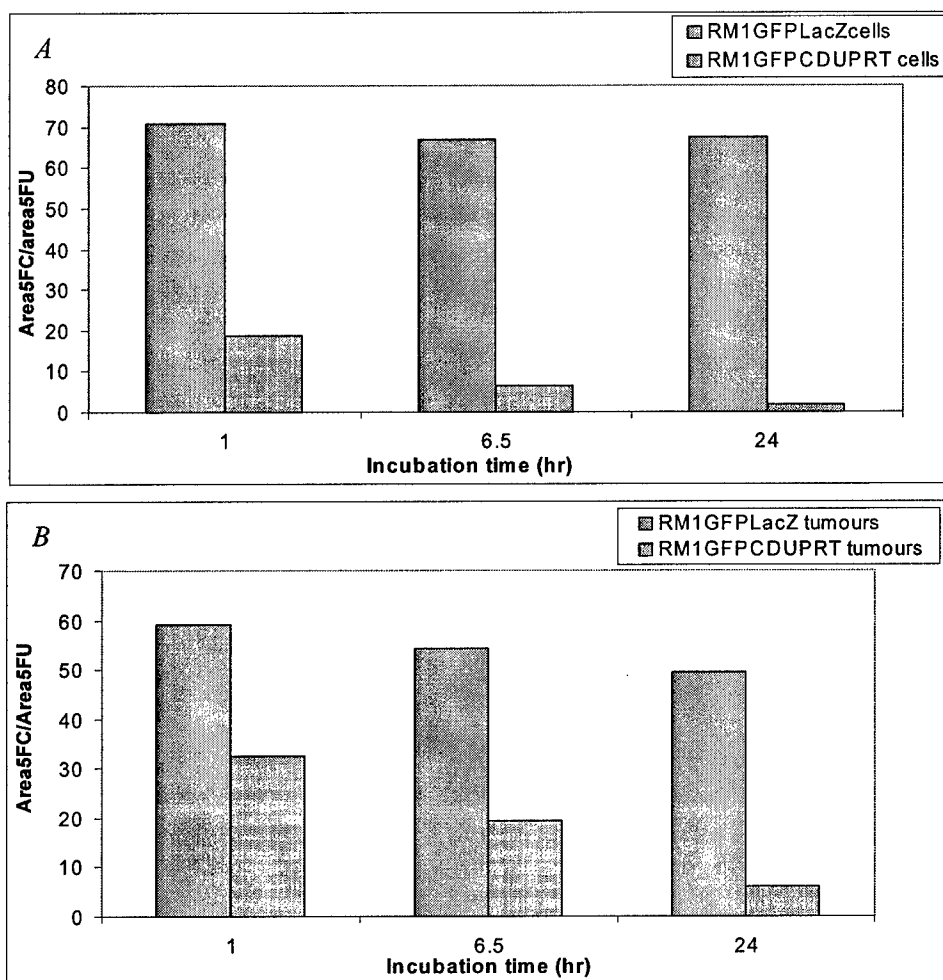


Figure 5: Characterisation of RM1-GFP/CDUPRT cells.

RM1-GFP/CDUPRT cells were tested for CDUPRT expression. The assay involved HPLC based evaluation of the capability of the cell/tumor lysates to catabolize the prodrug 5FC to 5FU. The enzyme activity for each sample was determined by ratios of the peak areas for 5FC and 5FU. Panel A shows the activity of the enzymes in lysates derived from RM1-GFP/CDUPRT cells *in vitro* and panel B shows the activity of the enzyme in lysates generated from the RM1-GFP/CDUPRT tumours grown subcutaneously in C57Bl/6mice. RM1-GFP/LacZ cells or tumours served as controls.

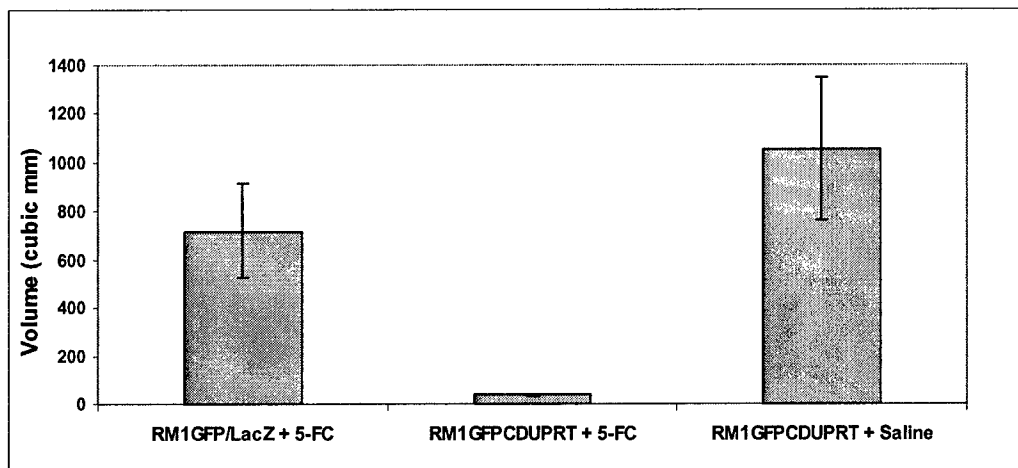


Figure 6: GDEPT effected by RM1CDUPRT cells *in vivo*.

5x10³ of RM1-GFP/CDUPRT (test) or RM1-GFP/LacZ (control) cells were implanted orthotopically in the prostate of C57BL/6 mice. 4 days post-implantation the prodrug 5FC (or saline, as a control) was administered intraperitoneally at 500mg/kg/mouse/day for 13 days. On day 19, mice were sacrificed and their prostate tumor volumes determined using the formula,

$$V = \pi/6(d_1 \cdot d_2)^{3/2}, \text{ where } d_1 \text{ and } d_2 \text{ are diameters at right angles.}$$

**H/E staining of paraffin-embedded intra-prostatic
RM1-CDUPRT tumor sections**

Saline



5 FC

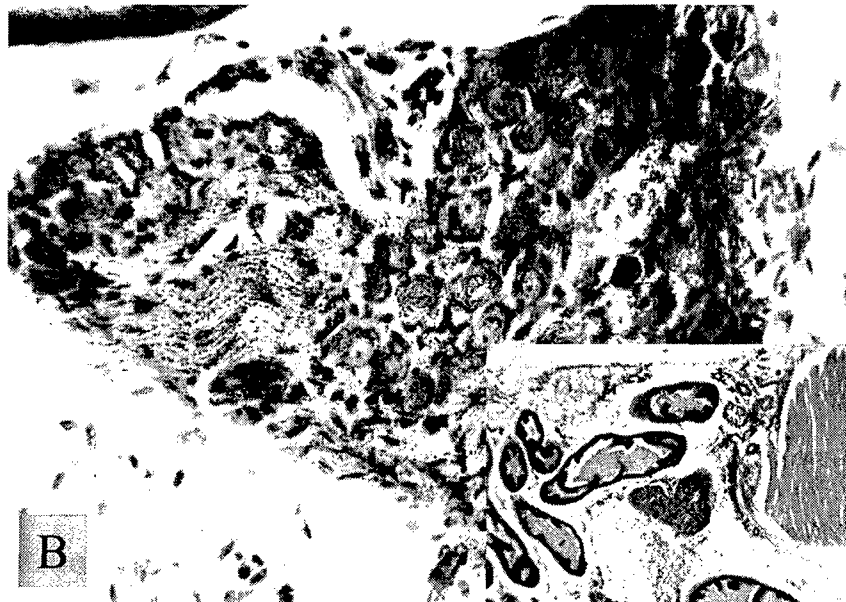


Figure 7: Effects of CDUPRT-GDEPT on histology of RM1 tumors

H &E staining of paraffin-embedded iprost RM1-CDUPRT prostate tumor sections. Treatment with saline resulted in highly vascularized viable tumor (A x40), whereas treatment with 5FC resulted in necrosis (B x40) with loss of prostate tissue architecture (insert, x10).

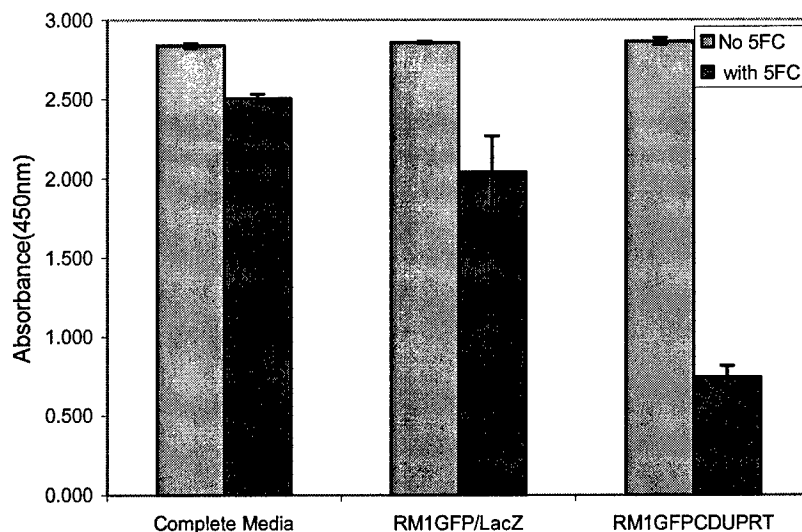


Figure 8A: *In vitro* evaluation of the local bystander effect of CDUPRT-GDEPT.

RM1-GFP/CDUPRT cells were grown in presence or absence of 5FC for 48 h, then the supernatants were collected. Supernatants from RM1-GFP/LacZ cells grown in the presence of 5FC, served as controls. Parental RM1 cells were then treated with these supernatants at 50% concentration. The bystander effect was demonstrated by cell killing of parental RM1 cells using the conditioned media from RM1CDUPRT cells.

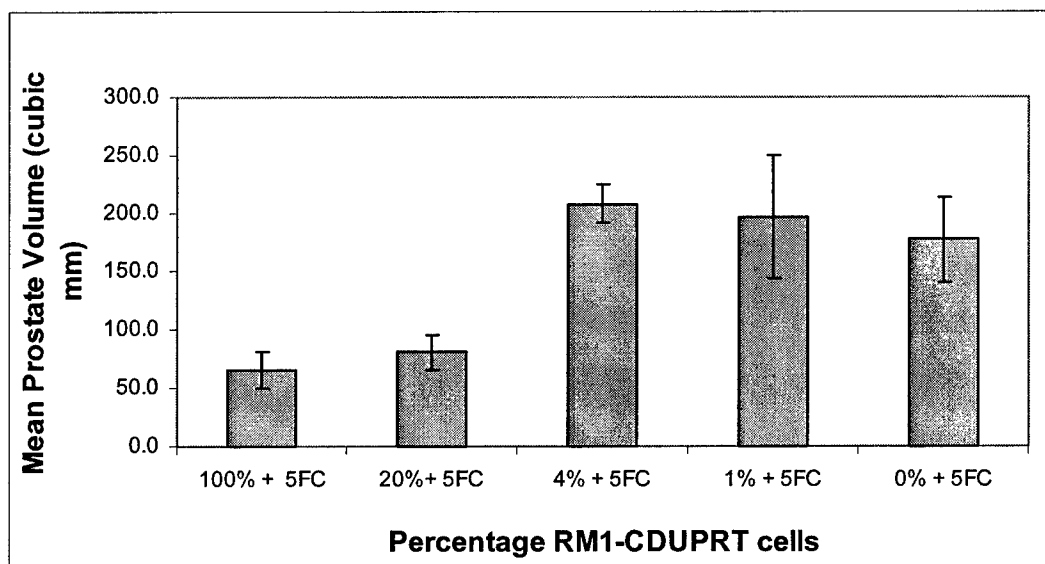


Figure 8B: Local bystander effect of CDUPRT-GDEPT *in vivo*.

RM1-GFP/CDUPRT cells were mixed with RM1-/GFP cells in different proportions (as shown) and a total of 5×10^3 cells were implanted in the prostate of C57Bl/6 mice. The mice were injected intraperitoneally with the prodrug 5FC at 500mg/kg/mouse/day from day 4 onwards daily for 15 days. Mice were sacrificed on day 19, and their prostate volumes determined using the formula,

$$V = \pi/6(d_1 \cdot d_2)^{3/2}, \text{ where } d_1 \text{ and } d_2 \text{ are diameters at right angles.}$$

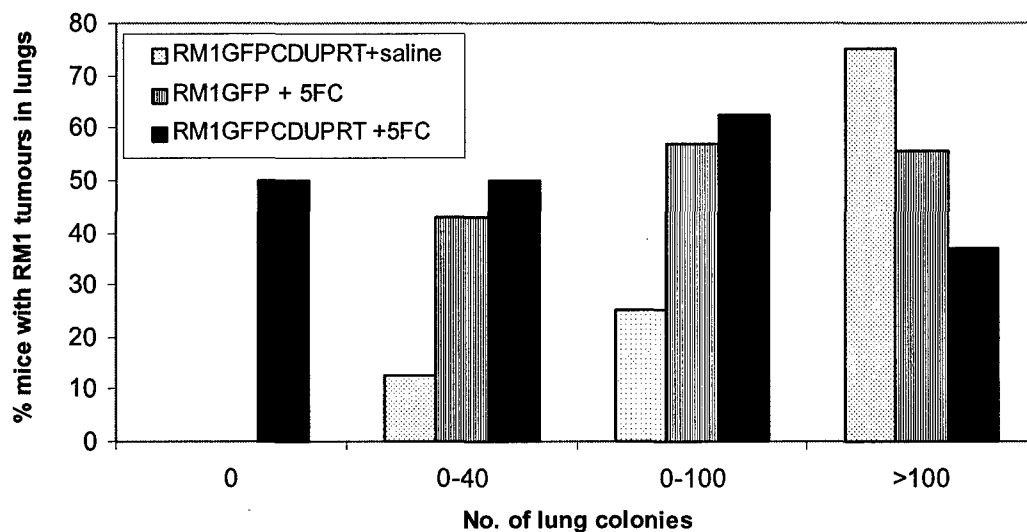


Figure 9: Distant Bystander effect of CDUPRT-GDEPT *in vivo*.

RM1CDUPRT (5×10^3) cells were implanted in the prostate of C57Bl/6 mice. Four days later, the mice were injected intravenously with the parental RM1 cells at the dose of 2.5×10^5 cells/mouse. The mice were injected ip with the prodrug 5FC at from day 4 onwards daily for 15 days 500mg/kg/mouse/day. On day 19, the mice were sacrificed and the lungs were harvested, stored in Bouin's reagent and colony counts were performed.

CD4

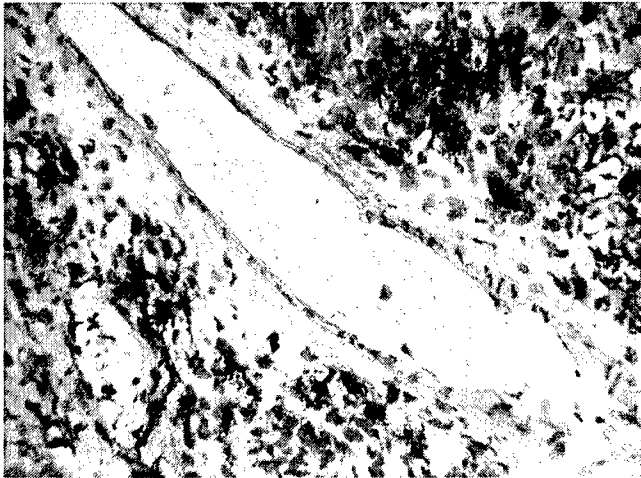


Fig. 10A Cluster of CD4 positive T cells (brown colour) in RM1-GFP/CDURPT+5FC tumor. 40x magnification
F4/80

CD

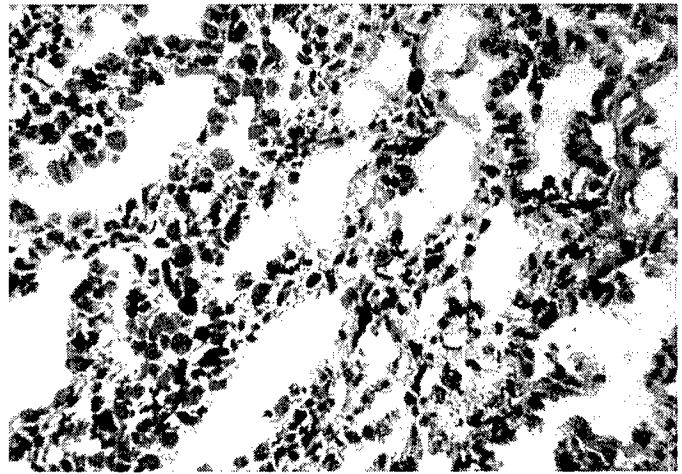


Fig. 10B Few CD4 positive T cells in RM1-GFP+5FC control tumor. 40x

AsialoGM

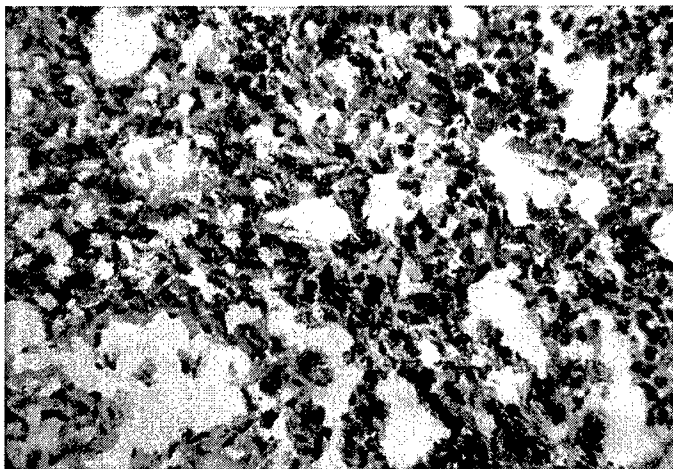


Fig. 10C Cluster of F4/80 positive macrophages in RM1-GFP/CDURPT+5FC

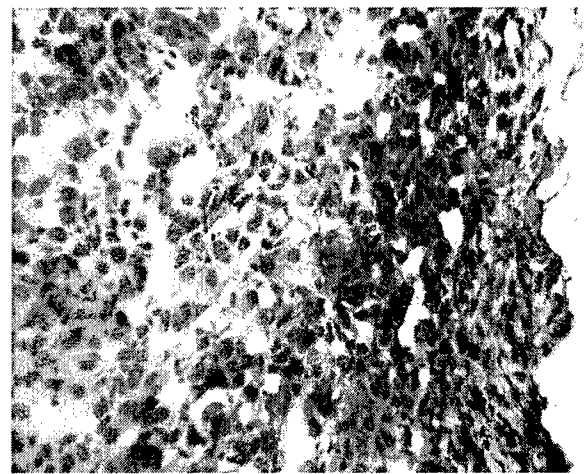


Fig. 10D AsialoGM1 positive immune cells clustered at the tumor periphery in RM1-GFP/CDURPT+5FC tumor. 40x

Figure 10: Intratumoral infiltrating immune cells following CDUPRT-GDEPT

Tumor angiogenesis

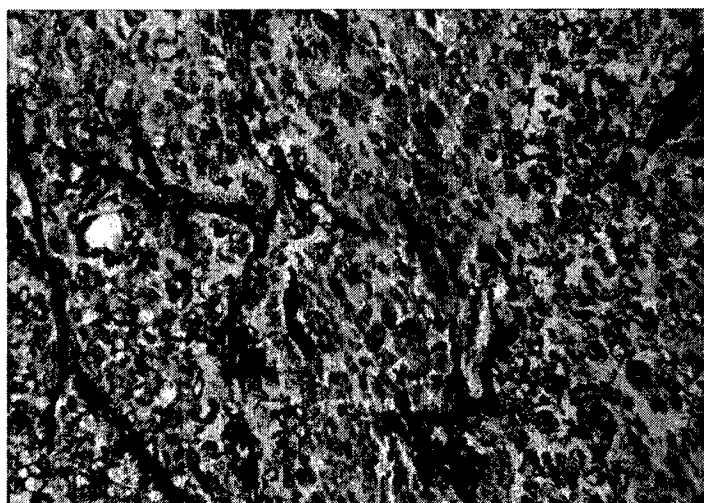


Fig. 11A CD31 positive endothelial cells (brown color) in microvessels of RM1-GFP/CDURPT+5FC tumor with decreasing vascularity. 40x magnification

Proliferation

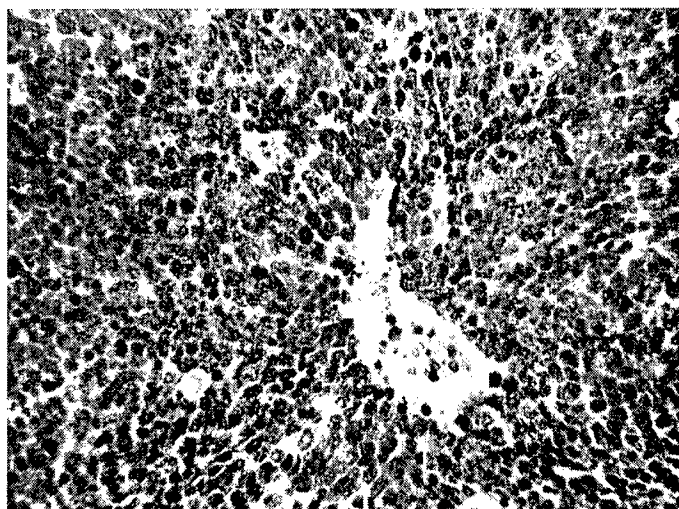


Fig. 11B Ki67 positive proliferating tumor cells (brown color) adjacent to blood vessel (v) in RM1-GFP/CDURPT+5FC tumor. 40x magnification

Apoptosis

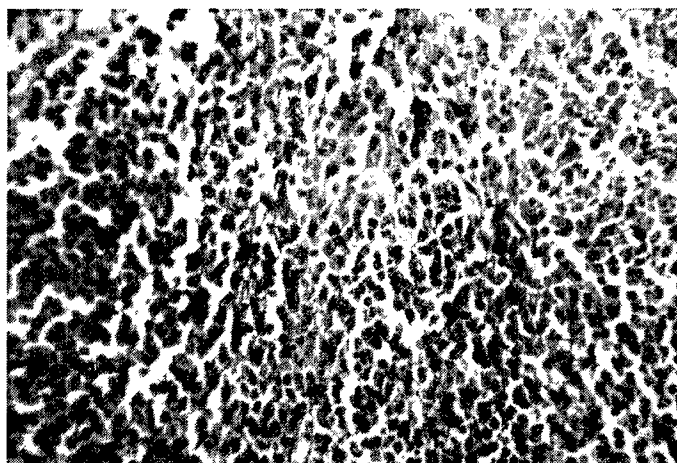


Fig. 11C Tumor cells becoming more apoptotic (brown color) further away from blood vessels: RM1-GFP/CDURPT+5FC tumor. 25x magnification

Apoptosis

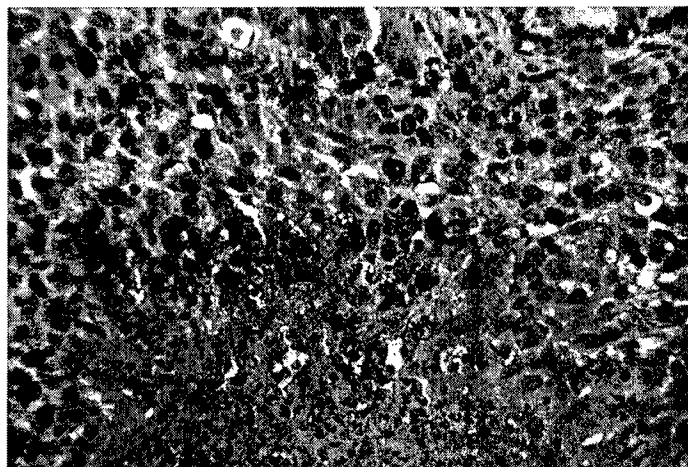


Fig. 11D A degenerative RM1-GFP/CDURPT+5FC tumor (pyknotic necrosis) showing numerous apoptotic tumor cells at its margin. Pyknotic cells were morphologically distinguishable from apoptotic cells though both stained brown in color using the TUNEL assay. 40x magnification

Figure 11: Effects of CDUPRT-GDEPT on angiogenesis, tumor cell proliferation and tumor cell apoptosis

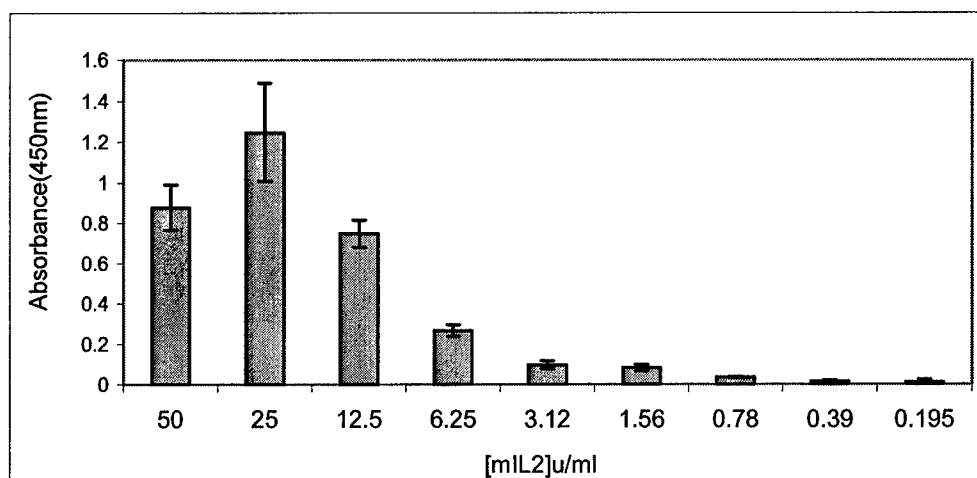


Figure 12: Proliferation of CTLL2 cells in response to mIL2 at 72 h post-treatment.

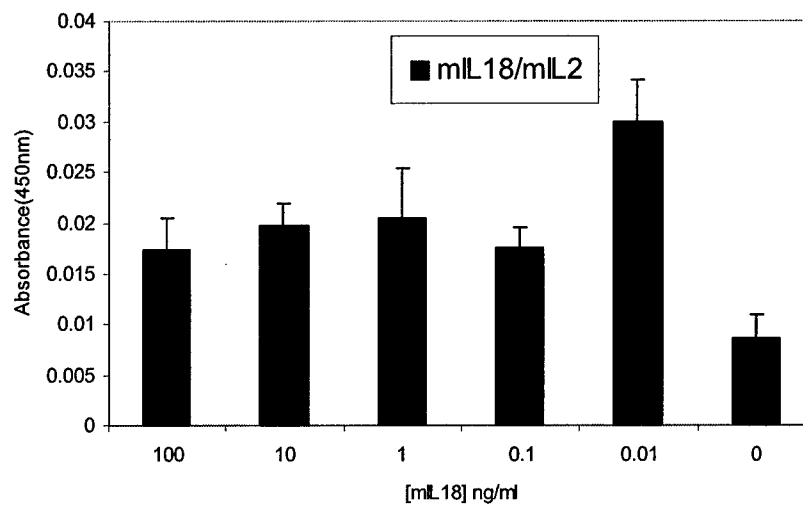
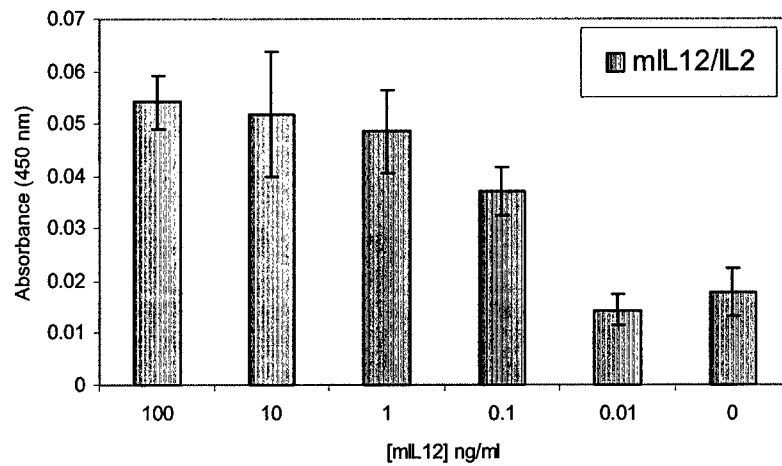


Figure 13 : CTLL2 proliferation in response to recombinant mIL12 (A) and mIL18 (B).

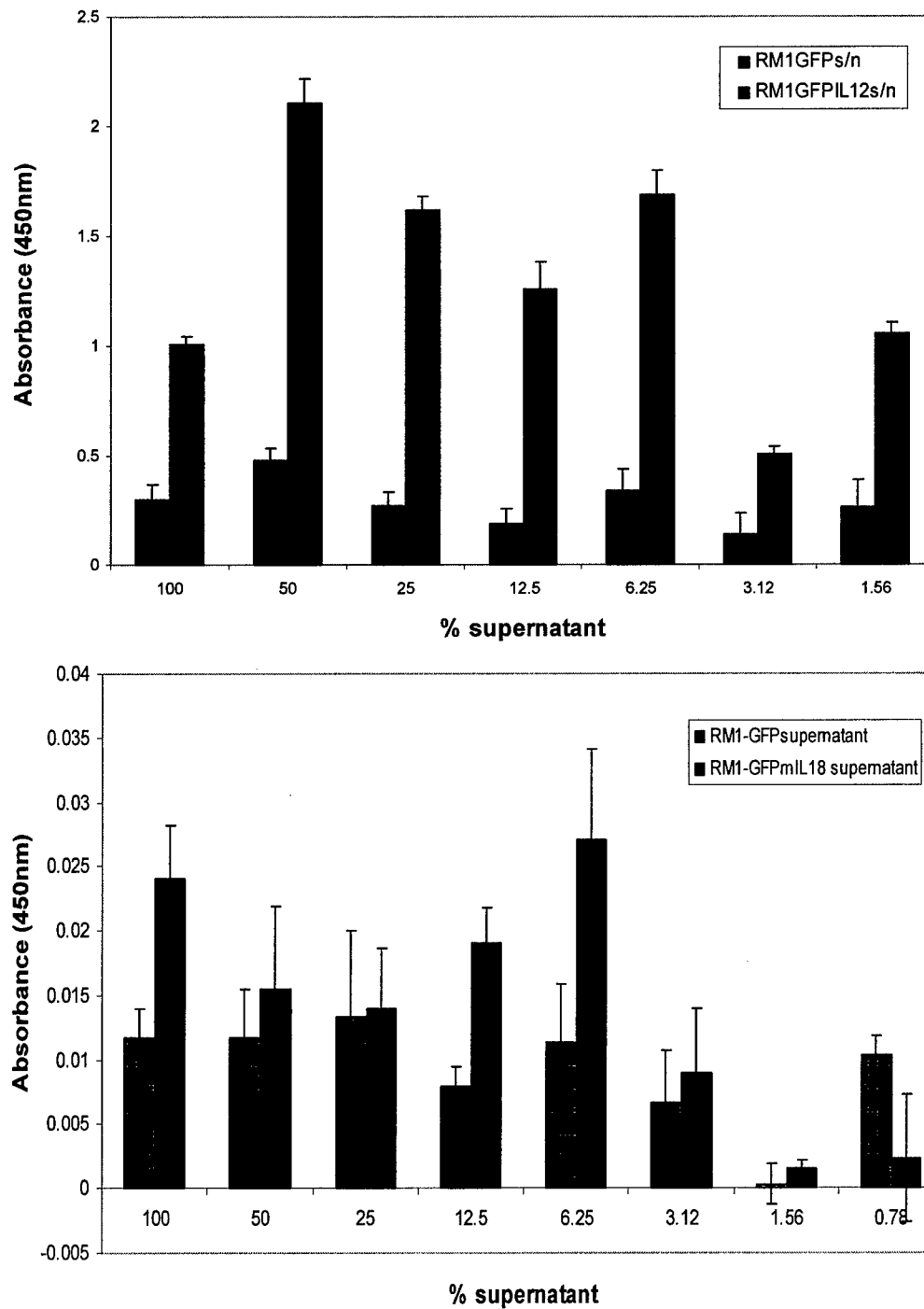


Figure 14: Effects of supernatants from RM1-GFPmIL12 (A) and RM1-GFPmIL18 cells (B) on CTLL2 proliferation. Supernatants from RM1-GFP cells were used as the controls.

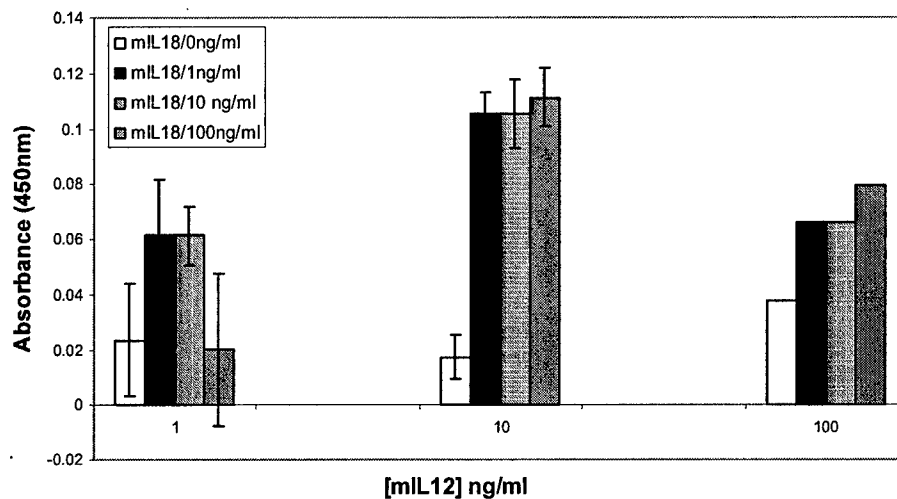


Figure 15: CTLL2 proliferation in response to combination of recombinant mIL12 and mIL18. Cells were incubated with mIL12 for 24 h followed by addition of mIL18, and then analysed for proliferation 48 h later. The data are normalized for no addition of mIL12 or mIL18.

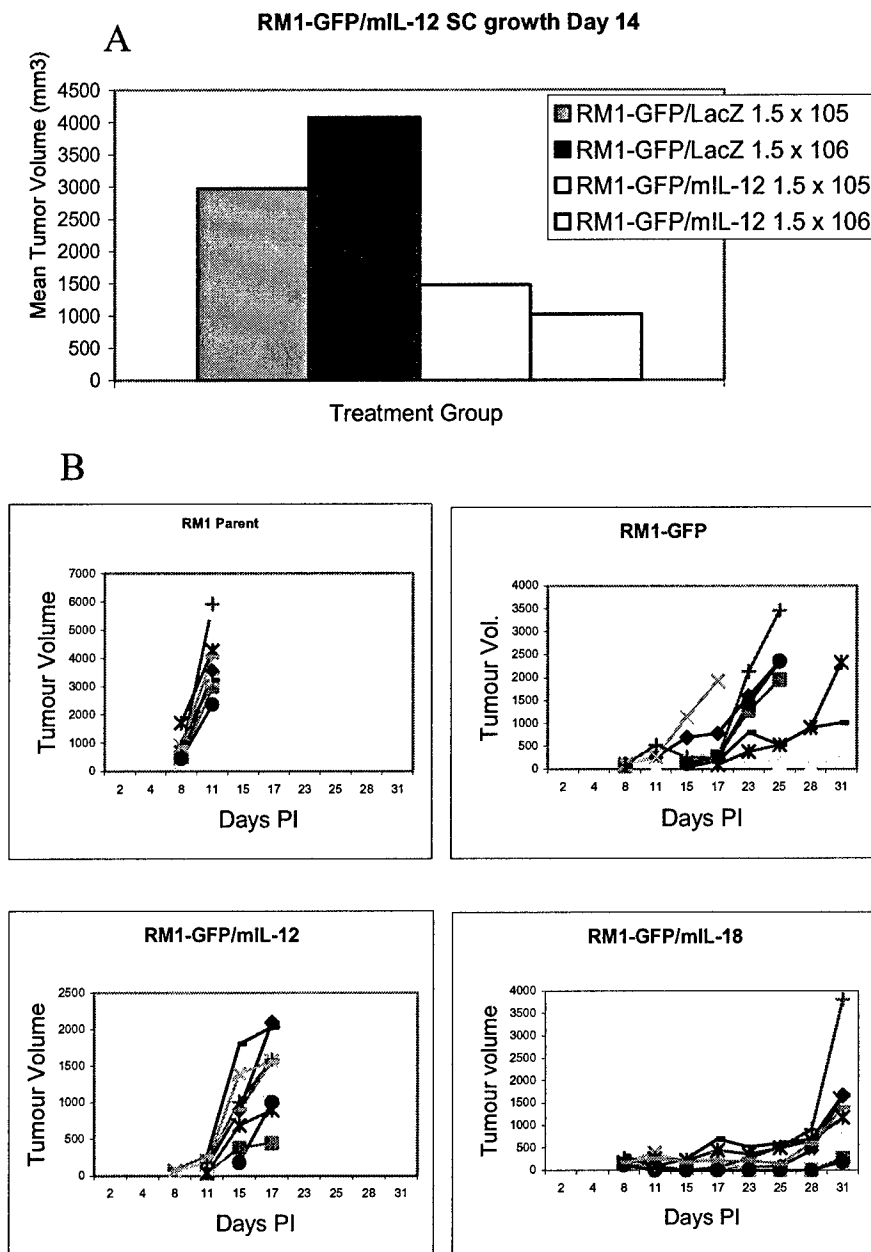


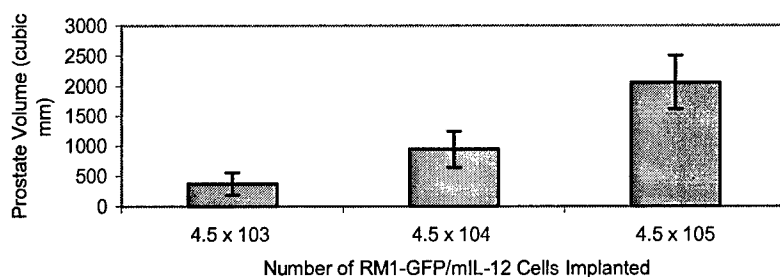
Figure 16: Growth of RM1-GFP/IL12 or RM1-GFP/IL18 cells sc in C57BL/6 mice.

A: shows mean tumor volumes indicating that higher numbers of RM1-GFP/IL12 cells grew to a smaller volume than those from lower numbers, suggesting that IL-12 secretion was slowing tumor growth.

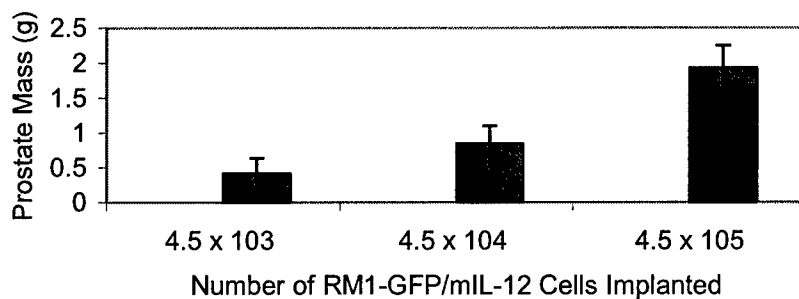
B: shows the tumor volume achieved in individual mice after injecting 3×10^5 cells sc.

PI = post injection.

A Prostate Volume vs Number of RM1-GFP/mL-12 Cells Implanted



B Prostate Mass vs Number of RM1-GFP/mL-12 Cells Implanted



C Prostate Volume vs Number of RM1-GFP/mL18 Cells Implanted in the Prostate

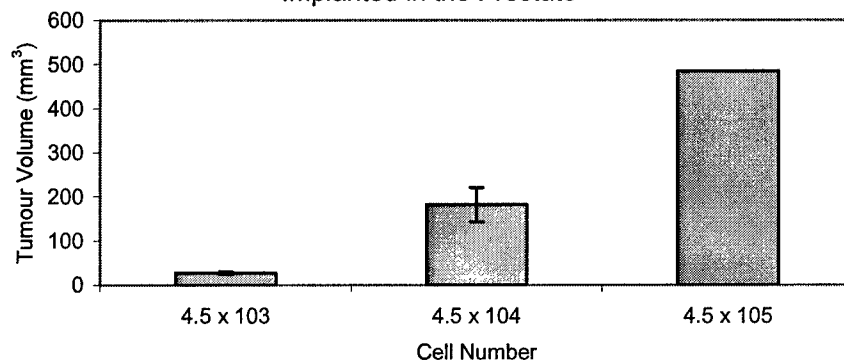


Figure 17: Relative growth of RM1-GFP/IL12 cells (A, prostate volume; B prostate mass) or C RM1-GFP/IL18 cells in the prostate of C57BL/6 mice after orthotopic implantation

Immunoreactivity to Asialo-GM1, CD8, CD90 and F4/80 of infiltrating immune cells in frozen tumor sections of iprost RM1-GFP (control cells) and RM1-GFP/IL12 cells

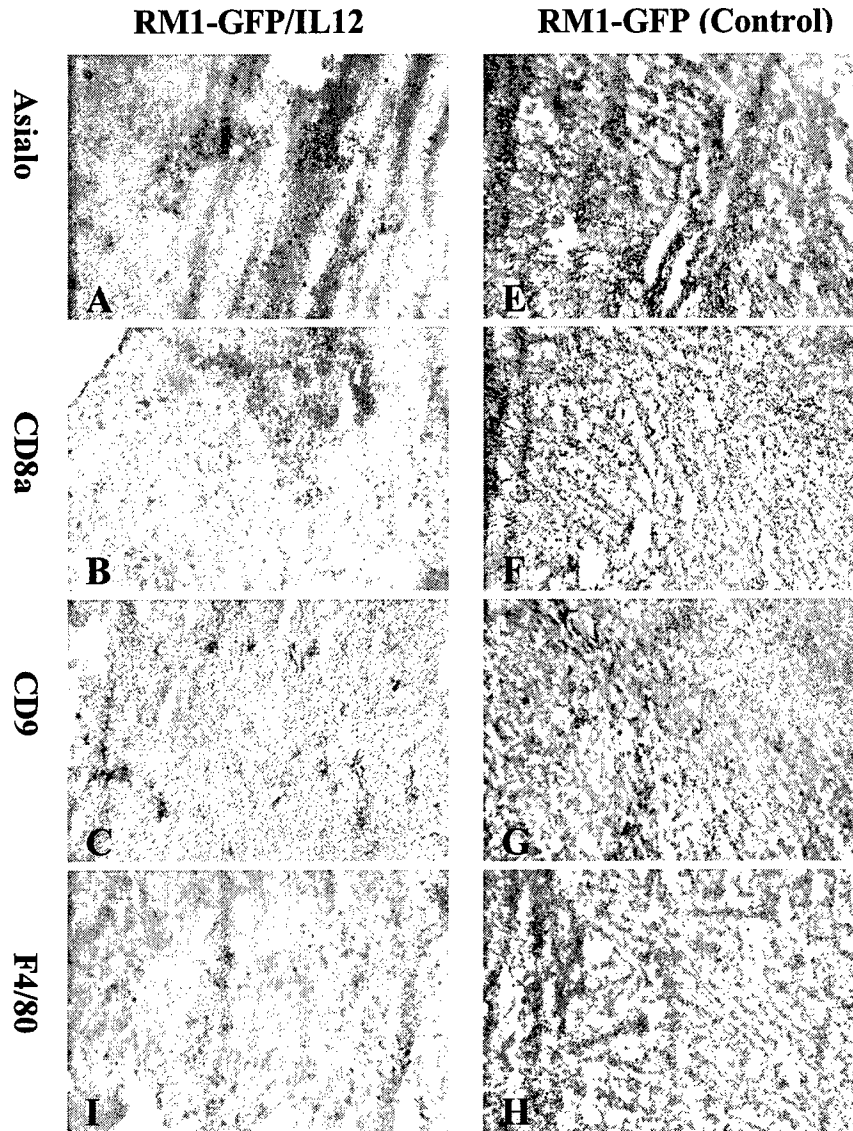


Figure 18: Photomicrographs of immune reactive cell infiltration (x10) in RM1 tumors expressing IL12.

Immunoreactivity (brown staining) to Asialo-GM1, CD8, CD90 and F4/80 of infiltrating immune cells in frozen tumor sections of RM1-GFP/IL12 cells (A-D) and RM1-GFP (control cells, E-H) grown in the prostate of C57BL/6 mice. A and E are stained for Asialo-GM cells; B and F for CD8a, C and G for CD90 and D and H for F4/80 positive cells. RM1 tumors expressing IL12 show increased infiltration of Asialo-GM+ (A) and CD90 cells (C) compared with control cells (E and G), as well as of CD8a (B) and F4/80 (D) positive cells that are absent in RM1-GFP tumors (F and H).

Appendix III

Abstracts:

1. E. Doherty, B. Curley, A. Khatri, N. Greenberg and P.J. Russell, Generation of an *in vivo* model for evaluation of prognostic, preventative and therapeutic strategies for late stage prostate cancer. TOW prize (Prince of Wales Hospital, September, 2004) (see poster attached)
2. Russell PJ, Khatri A, Zhang B, Doherty E, Ow K, Chapman J, Martiniello-Wilks R. Gene directed enzyme prodrug therapy using the fusion gene, cytosine deaminase uracil phosphoribosyl transferase leads to a distant bystander effect in mouse models of prostate cancer Submitted to AACR. 2005

Papers in preparation:

1. Khatri A, Zhang B, Doherty E, Chapman J, Ow K, Martiniello-Wilks R, Russell PJ. Local and distant bystander effects of CDUPRT plus 5-fluorocytosine gene therapy against RM1 prostate cancer cells in C57BL/6 mice. (to be submitted to Molecular Therapy)
2. Khatri A and Russell PJ. CTLL2 paper, A novel and convenient *in vitro* assay for functional analysis of murine IL12 and murine IL18 cytokines. (to be submitted to J Immunological Methods).
3. Khatri A, Curley B, Doherty E and Russell PJ. Generation of an *in vivo* model for evaluation of prognostic, preventative and therapeutic strategies for late stage prostate cancer.

Abstract 1:

GENERATION OF AN *IN VIVO* MODEL FOR EVALUATION OF PROGNOSTIC, PREVENTATIVE AND THERAPEUTIC STRATEGIES FOR LATE STAGE PROSTATE CANCER

E. Doherty, B. Curley, A. Khatri, N. Greenberg and P.J. Russell

Oncology Research Centre, Prince of Wales Hospital

Prostate Cancer is currently the most common cancer and the second highest cause of cancer death among men in Western society. Whilst early stage disease can be treated with traditional therapies (surgery, radiation and chemotherapy), metastatic prostate cancer is presently incurable. A relevant preclinical model that represents all stages of the human disease is required to evaluate potential therapies for these cancers. Although commonly used, transplantable syngeneic or xenogeneic murine models do not emulate the considerable biological and technical challenges inherent in cancer treatment. The transgenic adenocarcinoma of the mouse prostate (TRAMP) model closely mimics early stages of the human disease. However, this model does not adequately represent late stage androgen-independent, metastatic cancer.

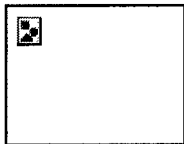
To rectify this we have developed potentially metastatic, androgen-independent derivatives of the TRAMP C1 and C2 cell lines to broaden the TRAMP model to include cell lines indicative of the various stages of prostate cancer progression. These androgen independent cell lines were established using two different approaches, an *in vitro* hormone deprivation of TRAMP C1 and C2 cell lines, and the *in vivo* derivation of an androgen independent TRAMP C1 cell population that grew in a female mouse. Characterisation of these cell lines for various markers of androgen independent, late stage metastatic prostate cancer and determination of their *in vitro* and *in vivo* growth characteristics is currently underway. This could potentially expand the TRAMP model to represent late stage androgen independent, metastatic disease and greatly facilitate the evaluation and development of future treatments for presently incurable late stage cancers.

1. Jemal, A., Tiwari, R.C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E.J. & Thun, M.J. (2004) *CA Cancer J Clin.* 54, 8-29

Abstract 2:

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Gene directed enzyme prodrug therapy using the fusion gene, cytosine deaminase uracil phosphoribosyl transferase leads to a distant bystander effect in mouse models of prostate cancer.

Short Title:

CDUPRT-GDEPT for prostate cancer

Pamela J. Russell, Aparajita Khatri, Bing Zhang, Eboney Doherty, Kim Ow, Jane Chapman, Rosetta Martiniello-Wilks. Prince of Wales Hospital, Randwick, Australia, Prince of Wales Hospital, Sydney, Australia, Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia

We are evaluating the therapeutic potential of gene directed enzyme prodrug therapy (GDEPT) using the fusion gene, cytosine deaminase uracil phosphoribosyl transferase (CDUPRT) for treating prostate cancer (PCa).

Objective: To test the efficacy of CDUPRT-GDEPT against RM1 mouse androgen-refractory PCa grown in C57BL/6 mice: RM1 cells were stably transfected with green fluorescence protein (GFP) and the fusion gene, CDUPRT, derived from *E coli* (RM1-GFP/CDUPRT). **CD/UPRT converts 5 fluoro-cytosine (5FC) to freely diffusible metabolites including 5-fluorouracil (5FU), that disrupt the metabolic pathways for both DNA and RNA synthesis, thus killing both dividing and non-dividing cells.** This is especially relevant to PCa, which is characterized by a low proportion of dividing cells.

Experimental Design: RM1 cells were stably transfected with plasmids containing GFP/CDUPRT, GFP or GFP/LacZ (controls) using lipofectamine. Cells that highly expressed GFP were selected by flow cytometry and used for further study. Transgene CDUPRT expression in cell lysates from cells grown *in vitro* or after *in vivo* implantation of RM1-GFP/CDUPRT was assessed by enzymic conversion of its substrate using HPLC. To assess the local bystander effect of CDUPRT-GDEPT, C57BL/6 mice were implanted directly into the prostate with cell mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions; 4 days later, 5FC was given intraperitoneally (ip) for 13 days at 500mg/kg/mouse/day. Pseudo-metastases in the lungs were established by a tail vein injection (iv) of untransfected RM1 cells 4 days post intraprostatic implantation. Mice were euthanased on day 19, and prostate weight and volume, and lung weight and colony counts were assessed. Tumors, lymph nodes, spleens and lungs were frozen or fixed for immunohistochemistry.

Results: Intraprostatic RM1-GFP/CDUPRT tumors on treatment with 5FC for 13 days resulted in complete regression of the tumors. Injection of cell mixtures (RM1-GFP/CDUPRT + RM1-GFP) resulted in a local bystander effect when only 20% of the cells were expressing the CDUPRT transgene. Interestingly, the lung colony counts indicated the presence of a distant bystander effect. The pseudo-metastases were absent in ~50% of mice in the RM1-GFP/CDUPRT+5FC group compared with the control groups. This is the first demonstration of a distant bystander effect using

CDUPRT-GDEPT.

Conclusions and future work: The CDUPRT GDEPT leads to a significant local and a distant bystander effect when used to treat androgen refractory RM1 tumors in mice. The role of the immune system in this distant bystander effect is currently under investigation.

Papers in preparation: (1)

Local and distant bystander effects of CDUPRT plus 5-fluorocytosine gene therapy against RM1 prostate cancer cells in C57BL/6 mice.

A.Khatri¹, B Zhang¹, E Doherty¹, J Chapman¹, K Ow¹, R.Martiniello-Wilks², PJ Russell¹

¹Oncology Research Centre, Prince of Wales Hospital Clinical School of Medicine, The University of New South Wales, Randwick, NSW, 2031, Australia; ²Rose's address

Running Title: CDUPRT-GDEPT using the mouse RM1 prostate cancer model

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KEYWORDS: GDEPT; CDUPRT gene; RM1 model; 5 fluorocytosine; local bystander effect

Abbreviations Used: AR, Androgen refractory; AS, Androgen sensitive; CaP, Cancer of the prostate; FAA buffer, 50% ethanol, 3.2% formaldehyde, 4% glacial acetic acid; FBS, Fetal bovine serum; GDEPT, Gene directed enzyme prodrug therapy; HSVtk, Thymidine kinase gene from Herpes Simplex Virus; OAdV220, Ovine atadenovirus vector expressing purine nucleoside phosphorylase (PNP) under the control of Rous Sarcoma Virus promoter; OAdV623, Ovine atadenovirus vector expressing PNP under the control of the PSMenhancer and Pb promoter; Pb, rat probasin gene; PNP, purine nucleoside phosphorylase; PSMA, Prostate specific membrane antigen; PSME, enhancer elements from prostate specific membrane antigen gene; RSV, Rous Sarcoma Virus; RTV, Relative tumor volume; VP, Viral particles.

Abstract

Purpose: To test the efficacy of gene-directed enzyme prodrug therapy (GDEPT) against mouse androgen-refractory (RM1) prostate cancer xenografts. RM1 cells were stably transfected with green fluorescence protein (GFP) and the fusion gene, cytosine deaminase uracil phosphoribosyl transferase (CDUPRT), derived from *E coli*, under the control of a cytomegalovirus (CMV) early promoter. CDUPRT converts a systemically administered prodrug, 5-fluorocytosine, to 5-fluorouracil that kills CDUPRT expressing cells, both non-dividing and cycling. Nearby cells are also killed via a bystander effect.

Experimental Design: RM1 cells were stably transfected with plasmids containing CMV-GFP/CDUPRT or with CMV-GFP/LacZ (control cells) using Lipofectamine 2000. Cells that highly expressed GFP were selected by flow cytometry and grown for further study. Transgene CDUPRT expression in cell lysates from cells grown *in vitro* or after *in vivo* implantation of RM1-GFP/CDUPRT or control cells in C57BL/6 mice was assessed by enzymic conversion of its substrate using HPLC-based methods. To assess the local bystander effect of CDUPRT-GDEPT, C57BL/6 mice were implanted directly into the prostate with cell mixtures of RM1-GFP/CDUPRT cells and RM1-GFP cells in different proportions on day 0 and 4 days later, 5-fluorocytosine treatment was given intraperitoneally for 13 days at 500 mg/m²/mouse/day. Pseudo-metastases in the lung were established two days after prostate treatment with transfected cells by a tail vein injection of untransfected RM1 cells. Mice were euthanased on day 18, and prostate weight and volume, and lung weight and colony counts were assessed. Tumors, lymph nodes, spleens and lungs were frozen or fixed in acetone for immunohistochemistry.

Results: After intratumoral administration of RM1-GFP/CDUPRT cells, treatment with 5FC for 13 days resulted in almost total obliteration of RM1-GFP/CDUPRT cell growth in the prostate. Injection of cell mixtures indicated that a local bystander effect was operating when 20% or more CDUPRT positive cells were present. Lung colony counts also indicated the effects of a distant bystander effect. Immunohistochemical studies showed an increase in immune cell infiltration by CD4 (but not CD8a) T cells, F4/80 macrophages and Asialo-GM1+ve NK cells. There was increased tumor necrosis and apoptosis and a decrease in tumor vascularity after CDUPRT-GDEPT.

Conclusions: CDUPRT-GDEPT significantly suppressed the very aggressive growth of murine androgen-refractory RM1 prostate cancer xenograft growth in mice by a mechanism involving both necrosis and apoptosis. This was accompanied by strong tumor cell infiltration of immune cells resulting in decreased lung pseudometastases.

Papers in preparation: (2)

A novel and convenient *in vitro* assay for functional analysis of murine IL12 and murine IL18 cytokines.

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Running Title: An in vitro function assay for murine IL12 and IL18.

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KEYWORDS: IL12, IL18, Functional assay, CTLL2 cells

Abstract

Purpose: To develop a convenient and accurate *in vitro* bioassay for testing the functionality of the cytokines murine IL12 (mIL12) and murine IL18 (mIL18).

The functional assays for both mIL12 and m IL18 are well established relying on the measurement of mitogen activated T cell proliferation in response to mIL12 or IFN γ release by activated T cells in response to mIL18. However, the problem is that both involve the use of animals to supply the lymphocytes (spleen and lymph nodes). To avoid the use of animals and for convenience, this *in vitro* assay was developed using the Cytotoxic T Lymphocyte Line (CTLL2) derived from C57BL/6 mice. We hypothesised that CTLL2 cells in the presence of baseline IL2, will respond to mIL12 and that this response will be enhanced when mIL12 and mIL18 are used together. This was on the basis of our observation that at least 60% of CTLL2 cells have murine IL12 receptors and that IL12 and IL18 act synergistically by upregulating each other's receptors (Ahn *et al.*,1997; Chang *et al.*,2000). This shared upregulation of receptors provides positive feedback mechanisms leading to synergism between the two cytokines.

Experimental Design: CTLL2 cells were grown in culture in the presence of a baseline level of IL2 that supported growth but not proliferation. They were then incubated with varying concentrations of mIL12 or mIL18 h either aloe or in combination and analysed for proliferation at different time intervals.

Results: CTLL2 cells showed a clear proliferative response to recombinant mIL12 or mIL18 in the growth media with up to 3.2 and 3.7 fold enhancement respectively compared with the controls (IL2 only). This was further confirmed when CTLL2 cells showed proliferation in response to supernatants from mIL12 and mIL18-expressing murine prostate cell lines but not from GFP expressing cells. The proliferative response to mIL12 containing supernatants was higher (~5 fold) than that to mIL18 supernatants (~2.5 fold). This could be due to a low percentage of mIL18 receptors on CTLL2 cells (10%). Further, the synergistic effects of the two cytokines were clearly demonstrated when CTLL2 cells were treated with recombinant mIL12 for 24 hours followed by mIL18 at different doses. Up to 10-fold enhancement in CTLL2 proliferative responses was observed with doses of mIL12 at 10ng/mL and mIL18 at 100 ng/mL.

Conclusions: These data suggest that IL2 responsive CTLL2 cells show clear proliferative responses to mIL12 and mIL18 either alone or when used together in tandem. The inhibition of the proliferative effects in the presence of the neutralising antibodies to the cytokines would confirm the specificity of the proliferative response. Once confirmed, this *in vitro* assay based on CTLL2 proliferative responses will provide an easy and accurate method of quantitative and qualitative assessment of mIL12 and mIL18 bioactivity.

Generation of an *in vivo* Model for Evaluation of Prognostic, Preventative and Therapeutic Strategies for Late Stage Prostate Cancer

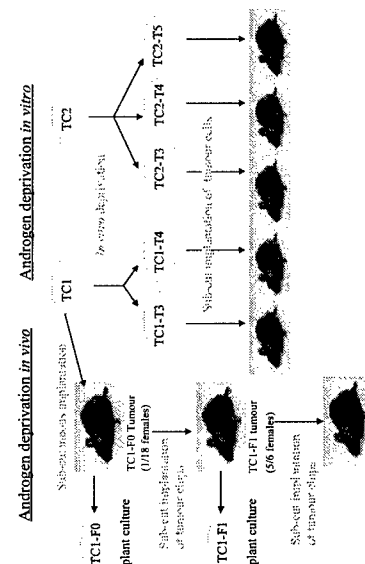
Eboney Doherty¹, Ben Curley¹, Aparajita Khatri¹, Kim T Ow¹, Pamela J Russell¹

¹Oncology Research Centre, Prince of Wales Hospital, Randwick, NSW and Faculty of Medicine, University of New South Wales, NSW, Australia,

Background

Prostate Cancer is currently the most common cancer and the second highest cause of cancer death among men in Western society. Whilst early stage disease can be treated with traditional therapies (surgery, radiation and chemotherapy), metastatic prostate cancer is presently incurable. Studies to evaluate potential prognostic, preventative and therapeutic strategies for prostate cancer have relied on transplantable syngeneic and xenogenic murine models. Although useful, these models only loosely resemble human disease and do not emulate the considerable biological and technical challenges inherent in its treatment. Transgenic mouse models of spontaneous prostate cancer overcome this as they mirror the disease in clinical setting. Indeed, the transgenic adenocarcinoma of the mouse prostate (TRAMP) model closely mimics prostate cancer. However, it does not adequately represent the later androgen-independent, metastatic stages of the human disease. Our aim was to rectify this by developing potentially metastatic, androgen-independent derivatives of TRAMP derived TRAMP C1 and C2 cell lines thus broadening the model to include cell lines indicative of the various stages of prostate cancer progression.

Methods



Potentially androgen independent cell lines were established *in vitro* through androgen deprivation of the cells. Dihydrotestosterone (DHT) was omitted from the standard culture media and the FCS added to the media was treated as detailed below.

Methods Cont.

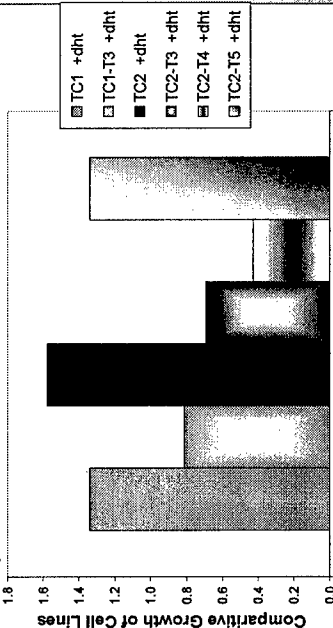
Treatment #	Charcoal Dextran Stripped (CDS) without Heat Inactivation	CDS with Heat Inactivated (HI)
Treatment 3	Yes	No
Treatment 4	No	Yes
Treatment 5	Yes	Yes

In vitro: The *in vitro* growth characteristics of the cell lines derived *in vitro* were determined using proliferation assays. The *in vitro* cell lines have been implanted sub-cutaneous in female mice to determine their *in vivo* growth characteristics.

In vivo: A potentially androgen independent cell line was derived *in vivo* by sub-cutaneous implantation of the TRAMP C1 cell line in 18 female mice. Only one of which developed tumour in 6 months. The *in vivo* growth characteristics of the tumour were determined by serial passaging of tumour pieces implanted sub-cutaneous into female mice. The morphology of the tumours was assessed by H & E staining.

Results:

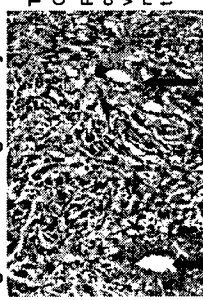
Figure 1 Androgen response of derived cell lines:
Proliferation of the parental and derived cell lines 48 hr post treatment with Dihydrotestosterone.



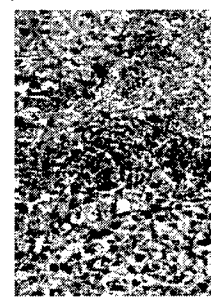
The data represented above indicates that, with the possible exception of the Treatment 5, each of the derivative lines is less responsive to testosterone than its parental line. Further *in vitro* investigations will aim to identify the cause of this altered androgen response.

Results

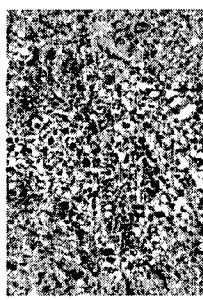
Figure 2 Histological analysis of F0 and F1 Tumours.



TRAMP C1 parental in Tramp+ Male
Growth Rate 21.2mm³ per day
Poorly differentiated cancer with a high degree of vascularisation (large blood vessels are indicated by arrows) Very little necrosis present in this tumour. The tumour had a firm structure upon excision.



TRAMP C1-F0 in Tramp- Female
Growth rate 4.7mm³ per day
Poorly differentiated cancer with minimal vascularisation. The tumour had a very firm structure upon excision. Moderately sized areas of necrosis are present throughout the tumour. The slow growth of this tumour is due to the time required for clonal expansion of androgen independent cells.



TRAMP C1-F1 in Tramp- Female
Growth rate 28.9mm³ per day
Poorly differentiated cancer with minimal vascularisation. The tumour had a very firm structure upon excision. Moderately sized areas of necrosis are present throughout the tumour. TRAMP C1-F1 xenografts established from TRAMP C1-F0 tumours have an accelerated growth rate in comparison with the parental line

Conclusions

Preliminary analysis of the cell lines derived from the parental TRAMP lines by *in vitro* androgen deprivation indicates that the cells are androgen independent but still androgen responsive implying that the androgen receptor in these cells may be functional. The androgen receptor status of the tumours which grew in female mice is yet to be determined.

Future Directions

To further characterise the new cell lines we plan to assess their *in vitro* growth characteristics with invasion, migration and proliferation assays. Tumours will be stained with antibodies against pan-cytokeratin to confirm epithelial origin and androgen receptor to test for a functional androgen receptor. *In vivo* growth properties of the tumours will be assessed by measuring the take and growth rate of the tumours as well as monitoring for metastases in normal and castrated mice.